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Analysis of phthalate esters, tin and organotin compounds and aquatic bioassays were conducted in the Chester River, a subestuary of Chesapeake Bay. No point source bioassays showed mortality of organisms. Analysis of phthalate esters in alluvial sediments showed a decreasing trend downstream from a point source effluent. Tin, microorganisms resistant to tin and microorganisms capable of transforming inorganic tin to organotin(s) were present at sampling sites. No significant field mortality of oysters were observed. Additional monitoring for phthalate esters is recommended.

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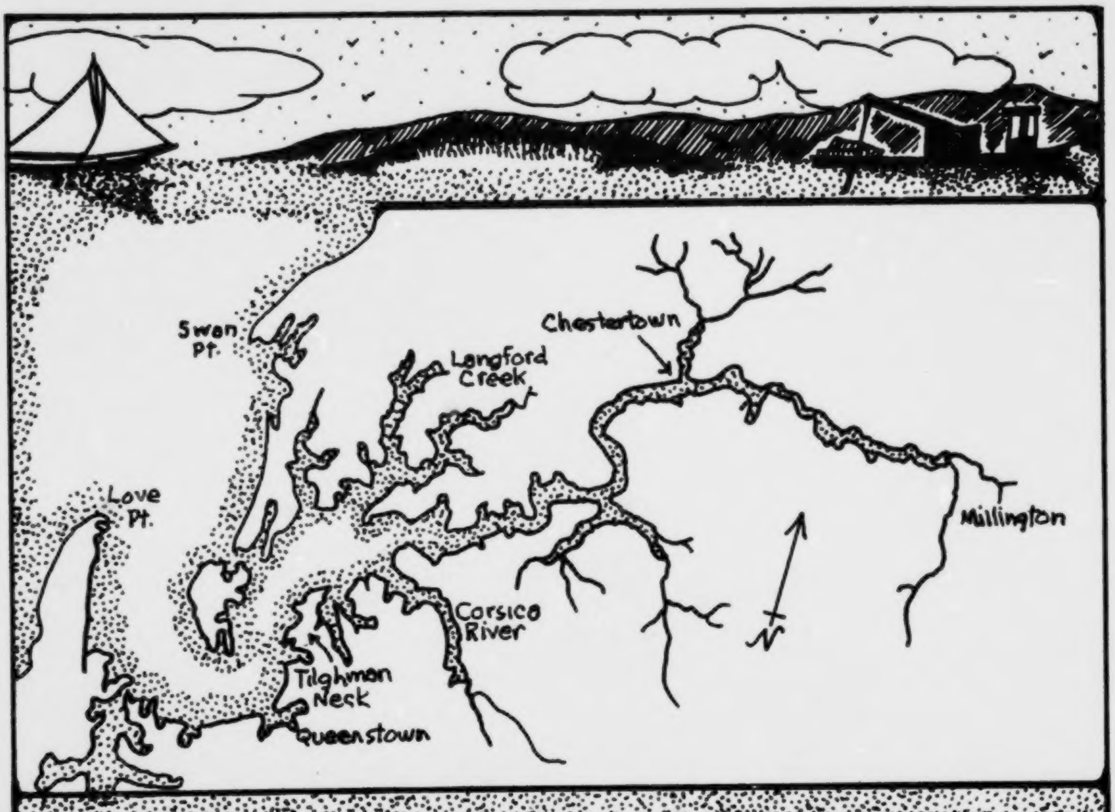

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AN EVALUATION OF CHESTER RIVER OYSTER MORTALITY

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PREFACE

The Maryland Water Resources Administration received a grant from the U.S. EPA Chesapeake Bay Program to conduct a study concerning oyster mortality in the Chester River, a subestuary of the Chesapeake Bay. The State of Maryland contracted with the University of Maryland to perform a three-part study to evaluate the reported oyster mortality. Due to the fact that this was a retrospective study, the evaluation of past large instananeous inputs of chemicals and the resulting potential impact was not made. The results of the University of Maryland's research follows.

The results of the three studies involving aquatic bioassays and analysis of phthalate esters, tin and organotin compounds in various environmental media did not show conclusive cause-and-effect relationships concerning the oyster mortality.

The bioassay study showed no significant point source or ambient acute aquatic toxicity to organisms tested; however, chronic stress was indicated by growth reduction of oysters during the study period and by occasional low levels of dissolved oxygen in the lower estuary. The point source bioassay observations showed mortality of organisms as well as controls; however, the controls and point source tests were not significantly different.

The analysis of phthalate esters in alluvial sediments showed a decreasing trend downstream from a point source. A pond receiving an industrial discharge showed extremely high concentrations of phthalate esters. The levels found in the vicinity of the pond warrant further consideration. Levels of phthalate esters in the estuarine sediments are low. The toxicity of the levels found is unknown, especially to oysters. A review of current literature shows no documentation of the toxicity and dynamics of phthalates to exposed oysters. The oyster concentration data in this report are the first published data available for di (2-ethylhexyl) phthalate (DEHP) in the American oyster. Although the toxicity of phthalates to the oyster would be expected to be low, because of the economic importance of the oyster, further laboratory bioassays are warranted to determine acute-and chronic-effect concentration levels.

Other modeling efforts conducted by state and Federal workers haveestimated that the major transport pathway of phthalate esters is in the dissolved fraction in the water column. It is not known at this time if the water phase transport of phthalates out of the system and other environmental degradation processes are great enough to preclude further buildup of DEHP in the estuarine sediments. Based on the levels of DEHP found in the pond receiving an effluent, further accumulation of phthalates in the estuarine sediments may be possible. Additional modeling could address these issues if sediment deposition and resuspension calculations were included in a modeling framework.

Tin, microorganisms resistant to tin, and microorganisms capable of transforming inorganic tin to organotin(s) were present at the sites sampled. However, the analysis of tin and organotin compounds in Chester River media did not result in any data to support or preclude the possibility of oyster mortality due to organotin compounds.

In summary, no significant mortality of oysters was observed during the course of this study. However, there are indications of chronic stress in the estuarine system based on the results of this study. Should mortality be observed again at any time in the future, it is recommended that oyster samples should be taken immediately, stored, and later analyzed for suspected xenobiotics. At the same time, water quality variables such as dissolved oxygen, salinity and other environmental variables should be observed as soon as possible after the mortality is reported. It should be noted that the greater the time period between when mortality of organisms is reported, and the analytical observations are made, the more difficult it will be to evaluate possible cause-and-effect relationships.

In addition, the acute and chronic effects of phthalate esters to estuarine organisms is relatively unknown. Additional monitoring of phthalates in this River is recommended as well as laboratory aquatic bioassay tests. Monitoring of oyster growth and mortality, dissolved oxygen and salinity over a several year period is required to determine if the observed high mortality has subsided. Such monitoring would help to identify causes of chronic stress indicated by reduced growth rate of the oysters.

ABSTRACT

EVALUATION OF CHESTER RIVER MORTALITY BIOTOXICITY

Three studies were performed to determine whether the recent dieoffs of oysters in the Chester River can be correlated with point sources of toxic substances. To this end two kinds of experiments were performed: long-term experiments with oysters placed in the Chester River; and 96-hour acute toxicity experiments with golden shiners, *Notemigonus chrysoleucas*, and crayfish, *Procambarus acutus acutus*.

In the long-term studies, 10 stations were established. Three stations were below the areas of known oysterkills, four were within the areas of recent oysterkills and three were in areas where the kills occurred in 1974 and 1975. One or two trays with 96 oyster specimens were placed at each station. Ten or twelve oysters were removed at nine intervals during 4 months. These were scored for condition. No significant mortality occurred during this period, but during July and August 1978 the five stations most upriver had dieoff of the fouling organisms and reduced growth rates of the oysters.

The 96-hour acute toxicity studies were performed by placing cages of golden shiners and crayfish in streams receiving effluents from the Campbell's Soup plant, the Tenneco, Inc. plant and the sewage treatment plant for the city of Chestertown. No significant mortality occurred.

No point sources of toxicants were located, but since no significant mortality occurred during the study these results are not conclusive.

PHTHALATE ESTERS AND RELATED CHEMICALS IN THE CHESTER RIVER BASIN

The Tenneco factory on Morgan Creek is permitted to discharge up to 2830 kg of organic extractables per year. These waste chemicals empty into the adjacent Tenneco Pond with a residence time of ca. 10^3 days. The pond discharge flows through Morgan Creek into the Chester River. The residence time in the river is a much shorter 130 days.

Model concepts based on available data allow plausible calculations of discharge organics in the contiguous downstream sediments. The possibilities range from 0.04 ppm based on simple

partition to 5 ppm for a "hot spot" model based on previous dye discharge experiments.

New chemical methods based on gas chromatography/mass spectrometry analysis of DBP (dibutylphthalate) and DEHP (di(2-ethylhexyl)phthalate) were developed. The relative standard deviation was demonstrated at ± 20 percent of the 0.1 ppm level in sediment. Accuracy is more difficult to specify, but this may be judged on the basis of split samples measured by an independent laboratory (0.3 ppm) and our own data (0.1 ppm).

MICROBIAL TRANSFORMATION OF TIN

This work was undertaken to determine if tin or products resulting from the biotransformation of tin may contribute to oyster mortality in the Chester River, Maryland.

Data were collected at two sites in the River: Spaniard Bar, which suffered extensive oyster mortality, and Buoy Rock, which did not exhibit extensive oyster mortality. Three sites associated with potential sources of tin in the River were also studied: the Tenneco plant and the Campbell's Soup plant, both near Chestertown, Maryland, and the Chestertown sewage treatment plant. For comparison, some samples were taken in Baltimore Harbor, a site known to be polluted with heavy metals, and in Tangier Sound near Tilghman Island, a site regarded as relatively free of pollution.

Water and sediment samples were examined for total viable counts of microorganisms, for counts of microorganisms resistant to inorganic tin, and for counts of microorganisms resistant to organic tin. Sediment from each site was used as inoculum for cultures to determine if microorganisms at the site could transform inorganic tin to volatile (organic) tin compound(s). Water and sediment were assayed for tin content.

Among physiochemical parameters measured onsite, only low dissolved oxygen is a potential contributor to oyster mortality. Microorganisms resistant to inorganic tin were detected in all samples and most samples contained microorganisms resistant to organotin, although organotin was more toxic than inorganotin to the microbial flora. Microorganisms capable of converting inorganic tin to volatile tin compound(s) were present at every site. Comparison of tin concentrations at the several sites showed that it is not possible to attribute the oyster kill solely to tin, although interaction with other stress factors is possible.

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David H. Freeman, Department of Chemistry, University of Maryland, College Park, Maryland, 20742, authored the study on Phthalate Esters and Related Chemicals in the Chester River Basin. The experimental work in this study was carried out by James C. Peterson and Sally A. Gingras. The work depended critically upon ultraclean glassware and John Trembly cooperated by facilitating routine use of the Chemistry Department's annealing ovens for this purpose. Mr. Arden Fox of Tenneco was helpful in providing details and insights to the history and present operations of the Tenneco plant and its bacterial waste processing facility. Captain O'Berry and the crew of the "Aquarius" ably assisted the collection of the Chester River water and sediment samples. The research sailing vessel "Huckleberry Friend" and the facilities of the Podickory Sailing Association were used to gather the reference samples from the mouth of the Chester River. Special thanks go to Dr. William Budde who generously provided cooperative measurements on split sediment samples. Finally, we happily acknowledge the value of Professor Ron Hites' intensive short course on environmental applications of GCMS. Drs. Nelson Frew, Robert Gagosian and John Farrington at Woods Hole Oceanographic Institution provided helpful consultation during the course of this work.

Microbial Transformation of Tin was under the direction of Joseph Cooney, Chesapeake Biological Laboratory, Center for Environment and Estuarine Studies, University of Maryland, Solomons, Maryland, 20688. Experimental work was conducted by L. E. Hallas, with the assistance of Marthe Cole-Jones and Terri Ekelund.

SECTION 1

INTRODUCTION

EVALUATION OF BIOTOXICITY

The Chester River is a tributary of the Chesapeake Bay, northeast of Kent Narrows. Historically, this estuarine part of the river has been rich in natural oyster bars; but, in the winter of 1974, a heavy mortality started upriver and moved over a period of years to bars in the lower reaches. The symptoms of this oyster mortality could not be attributed to any climatic or other natural phenomena that cause occasional oyster mortalities. A suspicion became plausible that something highly toxic to oysters had entered the upper river and had gradually worked its way downstream. A review of possible point sources of waste discharge into the Chester River system revealed the presence of the Chestertown sewage plant on Radcliffe Creek, the Campbell's Soup factory on Morgan Creek and the Tenneco plant that dumps its effluent into a pond which eventually empties into Morgan Creek via a small creek. The investigations reported here were aimed at locating point sources of toxic materials, and stations were chosen to maximize the ability to identify the roles of these potential sources.

PHTHALATE ESTERS AND RELATED CHEMICALS

The State of Maryland has issued a permit to Tenneco, Incorporated, in Chestertown, Maryland, to discharge 10 ppm of total organic extractables into Morgan Creek which empties into the Chester River and, then, into Chesapeake Bay. This permit would appear to be reasonably conservative unless it conceals the basis for long-range harm. Such threatening possibilities do exist and will be considered in the present studies. The major problem is related to the question of whether the discharge is free to dilute itself in some innocuous way, or whether, on the contrary, the dilution processes are blocked and the basis for a toxic accumulation can be shown. In the latter case, the permit would have to be viewed as nonconservative and the ecological threats would have to be carefully considered and perhaps further constrained.

The present study includes an initial investigation of various models for the distribution of the chemicals discharged from the Tenneco site. Various options are considered between the two extremes--that the organics are fully trapped at the Tenneco site, or that all the organics are distributed uniformly into the Chester River sediment beds.

The chemical analysis of sediment and oyster tissue for alkyl phthalates is prone to a myriad of error sources due to ubiquitous presence of these plasticizer compounds in the laboratory. As a result, there is a need to develop new technology that would scrupulously suppress the opportunity for contamination. The development succeeded because it relies on a well-established statistical maxim--that any risk if repeated often enough must lead to disaster. Since chemical methodology consists of a series of steps, each with an assigned risk, the approach was to develop a procedure that was designed to approach zero reliance on chemical manipulations.

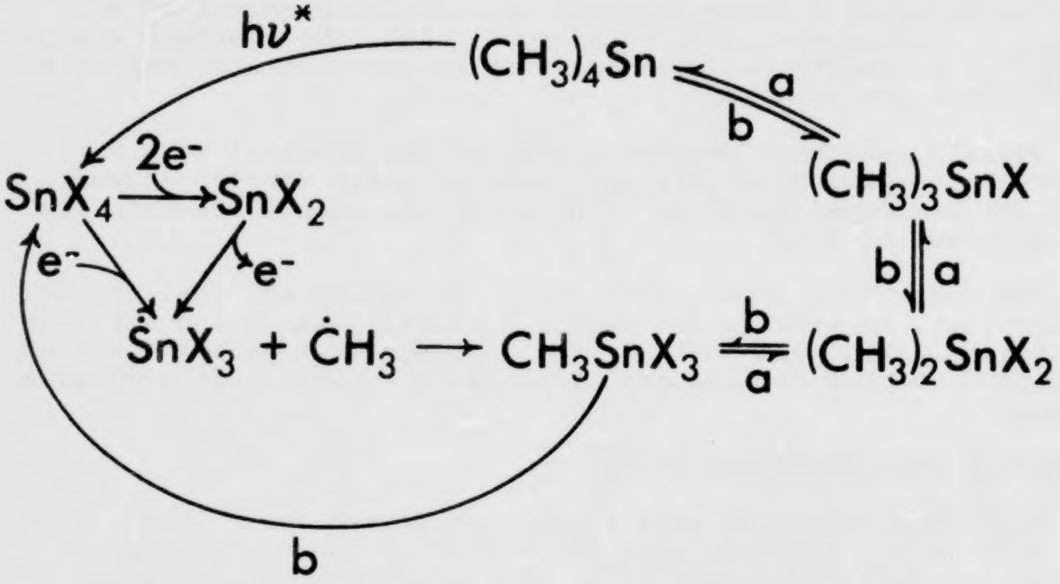
Quality assurance was not a part of the original work plan. However, independent laboratory tests of split samples showed that the developed chemical methodology was indeed adequate for the purposes at hand.

The underlying goals, then, were to develop and test the new methodology, to explore the possible causal link of industrially discharged alkyl phthalates to the past oyster mortality, and to determine whether the present discharge constitutes an ecological threat.

MICROBIAL TRANSFORMATION OF TIN

Organotin compounds were first synthesized about 1850 (Van der Kerk 1976), and they were first used as agents to control biological activity around 1930, when they were used as mothproofing agents (Luijten 1972). Shortly thereafter, organotins were used as stabilizers for vinyl resins, which continues to be a major application (Subramanian 1978). In the last 10 years, use of tin by industrial societies has more than doubled. Organotin compounds are used widely to control a variety of plants, animals, and microorganisms (Deschiens and Floch 1962, Daum 1965, Holden 1972, Luijten 1972, van der Kerk 1976). All such organotin compounds are toxic, but the effect varies with the organic group(s) present (Thayer 1974). In general, triorganotin compounds are more toxic than di- or tetraorgano compounds. Diorganotins behave like organomercurials, reacting with sulfhydryl groups to inactivate enzymes. Trialklyl tin compounds interfere with oxidative phosphorylation and with photosynthetic phosphorylation (Thayer 1974). Methyl tin compounds are poisonous to the central nervous systems of higher organisms (Ridley, Dizikes, and Wood 1977). Effects can be species-specific. For example, at concentrations too low (15-30 ppb) to affect some fish, triphenyltin acetate and several tributyltin compounds affect snails, zooplankton, and small fish, while warm-blooded species show no toxicity. In contrast, some soil microorganisms are not affected by concentrations of tributyltin oxide at concentrations up to 100 ppm (Thayer 1974).

In comparison with other metals such as mercury, lead, and cadmium, relatively little is known about biological transformations of tin. But sufficient information is available that a biological cycle has been proposed (Ridley et al. 1977).



$$\text{(a)}\ CH_3SnX_3 + e^- + \dot{C}H_3 \longrightarrow (CH_3)_2SnX_2 + X^-$$

In the diagram, X indicates a counteranion. The free radical SnX_3 can be methylated in a series of biological reactions (reaction a) involving methylocobalamins (e.g., vitamin-B_{12}) to yield mono-, di-, tri-, and tetramethyl tin (Ridley et al. 1977). A Pseudomonas species which is purportedly capable of methylating tin has been isolated from Chesapeake Bay (Huey et al. 1974). In the presence of ionic mercury, trimethyltin can react to yield the highly toxic methylmercury (Huey et al. 1974). Methyltins could be cleaved oxidatively (reaction b) by mixed function oxidases in the same way that trialkylyltin derivates are cleaved by liver microsomes (Kimmel, Fish, and Casida 1977). Alkyltins can also be degraded by photolysis (*) to yield free radicals (Lloyd and Rogers 1973).

Thus, tin can be transformed biologically to toxic compounds and these toxic compounds can be transformed chemically to other toxic compounds. Since tin is used at the Tenneco plant near Chestertown, it is a potential source of compounds toxic to oysters.

The objective of the present study was to determine if tin or products resulting from the microbial transformation of tin could contribute to oyster mortality in the Chester River. The investigation was not designed as a definitive study, but as a preliminary, screening study to determine whether further studies are warranted.

SECTION 2

CONCLUSIONS

EVALUATION OF BIOTOXICITY

Since there was no significant mortality in our experimental oysters, there was no strong indication that the causative factor for oysterkills in the Chester River was in operation during our studies.

Fouling organisms died off in July and August at five stations upriver from Corsica Neck. Since this accompanied dieoff of oysters in previous years, the phenomenon which is responsible for oyster dieoff might have occurred but in a mild form. Despite reasonable growth in the oysters planted in Chester River, the growth rate during the period of fouling community dieoff was significantly lower than that of controls placed in the Patuxent River. The phenomenon this year may have been so mild that the main effect in the oysters was a reduction in new growth.

The beginning of dieoff of the fouling community correlated with a fishkill. The fishkill originated well above Morgan Creek. The two phenomena may be unrelated as the fishkill may be bacterial in origin and species-specific since only carp and catfishes were noted dying.

Experiments with fish and crayfish in Radcliff Creek and Morgan Creek do not find any indication that either creek contains the sole source of the cause of oyster mortality within its drainage; however, the lack of significant mortality during the study period makes these results inconclusive.

PHTHALATE ESTERS AND RELATED CHEMICALS

The Chester River sediments taken from the vicinity of the oyster mortality zone, as well as farther downstream, show no evidence that Tenneco discharges are causally linked to the past oyster mortality. Oysters are now grown with apparent health in regions where the alkyl phthalates should be similar in concentration to those presently measured in the vicinity of the oyster mortality. Since the concentration history has not been measured, the study cannot rule out the possibility of a past causal relationship.

A massive buildup of alkyl phthalates manufactured by Tenneco was found in Tenneco Pond immediately adjacent to the factory waste water discharge plant. The levels show clearly that the pond sediments are serving as a sink, i.e., as a secondary waste treatment facility.

Experimental measurements of Chester River sediments show no significant differences between the mortality zone and the Chester River mouth where reasonably healthy oyster growth persists. The results for DBP and DEHP are very nearly identical in these regions in the Chester River: 0.02-0.85 ppm with average values of DBP (0.5 ppm) and DEHP (0.05 ppm). The DEHP/DBP ratio is 0.1 ± 0.07.

The Tenneco Pond results are quite different: DEHP (1.5×10^3 ppm) and DBP (0.2 ppm). Tenneco has rarely made DBP. The difference in the DEHP/DBP ratio alone suggests that the alkyl phthalates in the Chester River may originate from Tenneco as well as other possible sources. Moreover, the estimated possible accumulation in Tenneco Pond--10^3 kg of alkyl phthalates--suggests that the pond functions in part as a waste treatment facility.

The greatest threat seems to be that the Tenneco Pond may be nearing the saturation state that it must reach eventually. In that case, one can confidently forecast a serious accumulation of the alkyl phthalates that will in time spread out into the Morgan Creek area.

MICROBIAL TRANSFORMATION OF TIN

Examination of physiochemical data (pH, temperature, salinity, dissolved oxygen) from four estuarine and three freshwater sites shows only low dissolved oxygen near the bottom as a potential contributor to oyster mortality in the Chester River.

All sediment and water samples examined contain microorganisms resistant to inorganic tin; resistant organisms comprised as much as 55 percent of the total aerobic, heterotrophic population detected. Most of the water and sediment samples contained organisms resistant to the organotin compound, dimethyltin chloride; such organisms comprised as much as 17 percent of the total aerobic, heterotrophic population detected.

Microbial populations are more sensitive to organotin than to inorganic tin.

Microorganisms capable of converting inorganic tin to volatile tin compound(s) are widely distributed in the Chesapeake ecosystem.

All sediments associated with the Chester River--including sediments from the Tenneco plant, the Campbell plant, and the Chestertown sewage treatment plant--contained more tin than sediment from a site near Tilghman Island and less tin than a site in Baltimore Harbor. Spaniard Bar, which suffered an oysterkill, did not yield significantly more than Buoy Rock, which did suffer such a kill. Thus, it is not possible to attribute the oysterkill in the Chester River solely to pollution by tin, although interaction with other stress factors is possible. In addition to sediment, water in the Chester River and water entering the Chester River from the Tenneco plant, from the Campbell plant, and from the Chestertown sewage treatment plant sometimes contain significant quantities of tin.

Significant progress has been made toward developing a method for separation and qualitative and quantitative measurement of organotin species in environmental samples and in microbial cultures.

SECTION 3

RECOMMENDATIONS

EVALUATION OF BIOTOXICITY

Possible links between the dieoff of associated organisms and previous oysterkills should be further investigated. In addition to monitoring fouling communities of oysters, transite sheets should be placed on or near oyster bars and be sampled periodically. These sheets make quantification of the fouling organisms simpler and quicker when the effects are not gross.

The slower oyster growth in Chester River may be related to the oysterkill causal factor(s). Year-round sampling of oyster bars in the Chester River to monitor new growth is recommended. An area with a similar salinity regime and without such oysterkill phenomenon should be sampled simultaneously as a control. The studies recommended above could be performed concurrently.

A study continuously monitoring dissolved oxygen during periods when oysterkill may occur is recommended.

PHTHALATE ESTERS AND RELATED CHEMICALS

The levels of alkyl phthalates observed in the Chester River do not appear to present an immediate threat. However, the oyster lives a perilous existence. It is quite possible, and perhaps likely, that the alkyl phthalate levels are changing rapidly enough in the Chester River and the greater Chesapeake Bay to constitute a serious threat at some future time.

It is recommended that the rate of buildup be monitored through piston cores taken in well-stratified sediments. The goal should be to forecast the date when the extrapolated levels will be reached where healthy oyster production will be prevented by the presence of these ubiquitous chemicals.

It is recommended that the Morgan Creek sediments be surveyed for evidence of alkyl phthalate accumulation. The creek serves as a conduit between the Tenneco pond and contiguous farmland using the discharged water for irrigation purposes. The Tenneco Pond sediments are likely to be weakening in their role as a sink for these chemicals. Eventually, their sorptive capacity is likely to become exhausted.

It is recommended that Tenneco Pond be surveyed to establish the total phthalate plasticizer content. At the same time, cooperative research by Tenneco should be elicited to see if a more effective use of secondary waste water treatment by certain soils could be established in order to furnish long-range protection to the downstream ecology.

MICROBIAL TRANSFORMATION OF TIN

Tin should not be considered as the sole source of the extensive oyster mortality observed in portions of the Chester River.

Tin should not be excluded as a partial cause of oyster mortality in the Chester River, particularly when coupled with other pollutants and with low dissolved oxygen.

When tin enters an aquatic ecosystem, it should be assumed that microorganisms are present which can convert it to volatile tin compounds.

Studies should be undertaken, using recently developed methodology, to determine if oysters bioaccumulate tin and if the oyster's gut flora can produce significant quantities of volatile tin compounds.

SECTION 4

EVALUATION OF CHESTER RIVER OYSTER MORTALITY BIOTOXICITY

METHODS AND MATERIALS

Possible Sources of Toxicants

The Chester River has a typical estuarine portion with widely varying temperature and salinity regimes. All of the possible sources of toxicants that were examined have their waste materials entering the river in its tidal portion. These possible sources dump materials of widely differing nature. The Chestertown sewage plant discharges chlorinated and treated domestic sewage. The Campbell's Soup factory sprays its effluent, which are wastewaters from preparing chicken, over percolation fields and each leached water discharge is chlorinated before it enters into Morgan Creek. Tenneco does not discharge any effluent that can be made more acceptable by chlorination, as its effluent is resultant from manufacture of plasticizers. Instead of chlorinating, the factory discharges into a leaching pond at one end with a stand pipe overflow on the far end. The volume of effluent at the time of the study was a small volume compared to both the sewage plant and Campbell's Soup plant.

The proposal called for two separate but related studies: (1) a field study on mortality and growth of oysters in the Chester River, and (2) a field study to detect possible toxic effects on test animals by the effluents of the three plants discharging into the Chester River basin.

Oyster Studies--

The oyster portion of the study was conducted in the portion of the river from just below Chestertown to just above Kent Narrows, which is near the mouth. There were 10 stations in the Chester River from 15 May 1978 until 18 September 1978. Fifteen trays, each containing 96 oysters, were monitored for mortality. Qualitative information on oyster communities' associated organisms were also recorded.

Stations--See Figures 1 and 2 for orientation of stations. Comments concerning these stations are given below.

Station 1.	North of Kent Island, a single-tray station. This tray was attached to parts of a sunken barge. At the first sampling visit the tray had been tampered with and when retrieved it came up upside down with the lid open. No oysters could be recovered and the station was abandoned.
Station 2.	South of Cedar Point. This was a two-tray station attached to clam buoy "SS." This station lasted from 15 May until 27 June but could not be found with the grapnel nor by diving on 12 July.
Station 3.	Off Tilghman Creek. This single-tray station was tied to a stake at 0.6 m depth. This station lasted the entire study period.
Station 4.	Off Piney Point. A double-tray station tied to clam buoy "C." This station served from 15 May - 4 August but could not be found on the 23 August check and thereafter.
Station 5.	At Ringgold Point, a single-tray station, tied to a Coast Guard day marker by a nylon line to a ring on this structure. The line was cut by vandals before this station was visited on 27 June. The station was observed from 15 May - 14 June.
Station 6.	Off Corsica River. This station held two trays and was fastened to clam buoy "A." The tieline was attached to the buoy's anchor chain by means of a chain ring that dropped to the bottom. This station lasted the entire study period from 15 May to 18 September.
Station 7.	Nichol's Point, a double-tray station. This station was tied to a ring on a day marker. It was in service for the entire 15 May - 18 September period.
Station 8.	Off Cliffs Point, a single-tray station attached to the remnants of a booby blind in about 1 m of water. The station lasted the entire study period.
Station 9.	Off the mouth of Shippen Creek. This single-tray station was attached to a privately owned pier and was not tampered with.
Station 10.	Newman's Wharf just off Deep point. This two-tray station was also suspended from a private pier and lasted the entire study period.

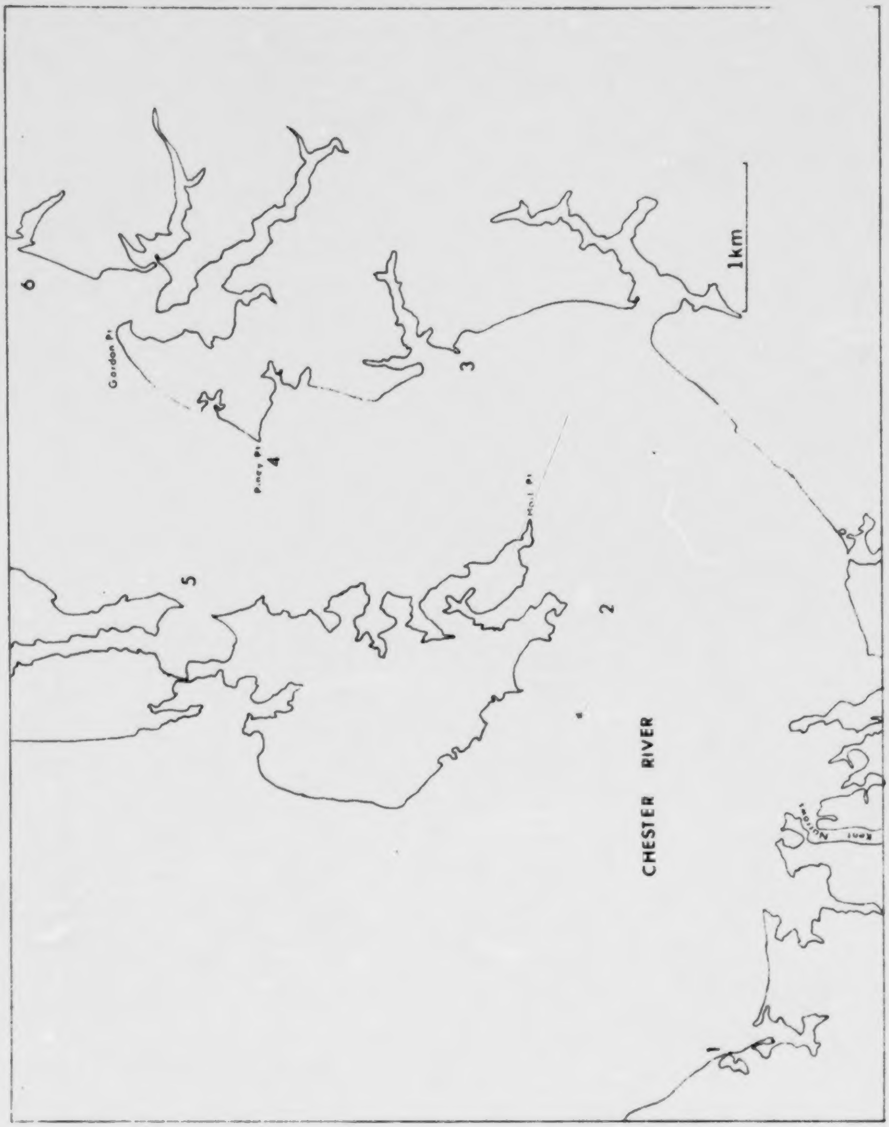


Figure 1. Lower Chester River showing oyster tray stations 1 thru 6.

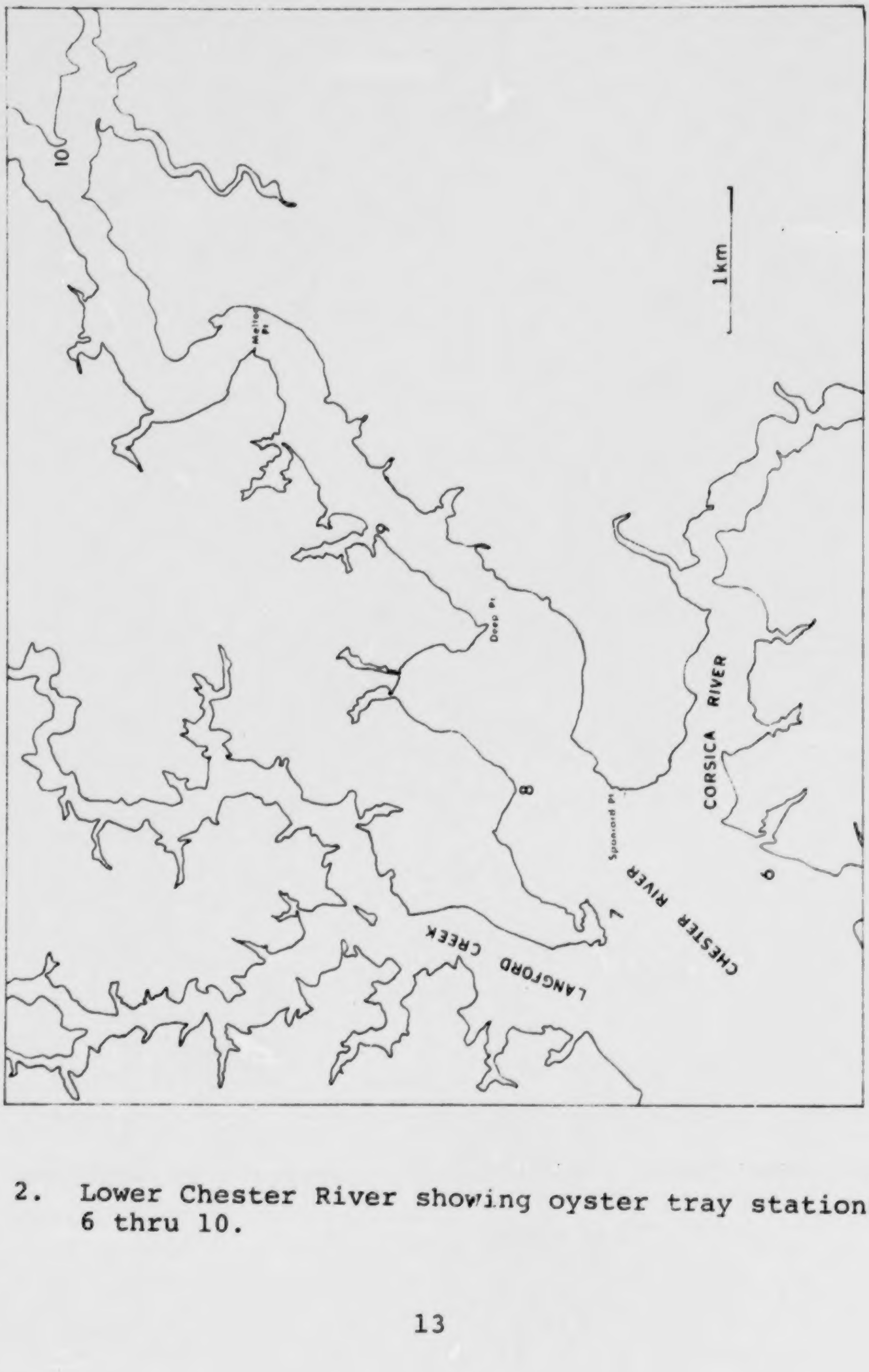


Figure 2. Lower Chester River showing oyster tray stations 6 thru 10.

Hydrographic observations of all stations are contained in Table 1.

TABLE 1. PHYSICAL PARAMETERS MEASURED AT CHESTER RIVER OYSTER STUDY SITES

	Depth (m)	Surface/Bottom			Secci Depth (m)
		Salinity (ppt)	Temperature (°C)	Dissolved O_2 (ppm)	
STATION 1					
15 May	1.5	7.6/7.4	14.0 /14.1		
23 May		5.5	18.0 /18.0	9.7 / 9.5	1.0
2 June					
14 June					
27 June					
12 July					
4 August					
23 August					
18 Sept.					
STATION 2					
15 May	3.3	7.1/7.1	14.3 /14.5		
23 May		5.5	20.0 /19.0	9.9 / 9.2	0.8
2 June		5			
14 June					
27 June		6.3	25.3		1.3
12 July		7.5/7.5	25.0 /22.5	6.9 / 6.5	
4 August					
23 August					
18 Sept.					
STATION 3					
15 May	1.5	6.9/7.1	14.1 /13.8		
23 May		5.8	24.0 /24.0	9.3 / 9.8	0.5
2 June		5			
14 June		5	21.3	7.8	0.6
27 June		6.5	26.5		1.1
12 July		7.0	24.7		
4 August		8.0	25.8	5.5	
23 August		10.0	27.2	6.8	
18 Sept.		13.0			

(continued)

TABLE 1. (continued)

	Depth (m)	Surface/Bottom			Secci Depth (m)
		Salinity (ppt)	Temperature (°C)	Dissolved O_2 (ppm)	
STATION 4					
15 May	3.6	6.6 /6.8	15.1 /14.6		
23 May		5.2	19.5 /19.0	11.4 / 8.4	0.5
2 June		6			
14 June		5	20.8	7.5	0.6
27 June		6.2	28.0		0.8
12 July		6.2 /6.2	24.7 /23.5		
4 August		7.0	26.1 /25.8	7.4 / 5.6	
18 Sept.					
STATION 5					
15 May	3.0	6.0 /6.0	15.3 /15.3		
23 May		5.8	21.0 /19.5	9.5 / 9.6	
2 June					
14 June		5.0	21.0	8.4	0.9
27 June					
12 July					
4 August					
18 Sept.					
STATION 6					
15 May	3.6	5.8 /6.5	15.3 /14.8		
23 May		5.9	19.0 /19.0	9.3 / 7.8	0.6
2 June					
14 June		5.5	21.0	8.0	0.7
27 June		6.0	28.2		0.6
12 July		7.2 /6.8	25.2 /24.4		
4 August		6.2	26.2 /25.9	5.9 / 4.7	
23 August		8.0	28.8 /26.6	7.0 / 5.4	
18 Sept.		9.0			
STATION 7					
15 May	2.4	5.4 /5.4	15.3 /15.3		
23 May		5.5	19.0 /18.6	8.2 / 7.7	
2 June					
14 June		5	22.1	7.2	0.7
27 June		6.0	28.3		0.7
12 July		6.0 /5.9	25.2 /25.8		
4 August		6.0	26.2	5.9	
23 August		8.0	27.4 /27.0	6.8 / 5.8	
18 Sept.		8.0			

(continued)

TABLE 1. (continued)

	Depth (m)	Surface/Bottom			Secci Depth (m)
		Salinity (ppt)	Temperature (°C)	Dissolved O_2 (ppm)	
STATION 8					
15 May	3.4	5.0 / 5.0	15.2 / 15.2		
23 May		4.8	18.0 / 19.0	9.2 / 8.3	
2 June		6			
14 June		5	22.0	6.9	0.6
27 June		5.5	28.0		0.5
12 July		5.8	26.2		
4 August		6.0	26.5	5.4	
23 August		8.0	27.8 / 27.0	6.5 / 5.4	
18 Sept.		7.0			
STATION 9					
15 May	4.3	4.4 / 4.2	15.3 / 15.1		
23 May		4.5	18.5 / 18.5	8.2 / 8.0	
2 June		6			
14 June		4	23.0	6.8	0.4
27 June					
12 July		5.0	26.0		
4 August		5.8	26.5	5.8	
23 August		7.0	27.8 27.0	5.8 / 5.2	
18 Sept.		9.0			
STATION 10					
15 May	14.3	3.3 / 3.3	15.7 / 15.7		
23 May		3.0	19.0 / 19.0	8.0 / 7.5	
2 June		2			
14 June		2	22.5	7.5	
27 June					0.2
12 July		2.2	26.2		
4 August		3.0	26.9	4.8	
23 August		5.0	27.5	6.0 / 5.6	
18 Sept.					

PROCEDURES

Oysters used in the field experiment had to be obtained from a source that was free of phthalic esters, therefore, the natural oysters in the Chester River could not be used. Cultchless oysters with a height of approximately 3 cm were bought from Frank Wilde's Hatchery in Shadyside, Maryland. The oysters were distributed randomly over 15 trays with 96 oysters per tray. Trays were of stainless steel and measured 40 cm x 92 cm x 10 cm with a mesh of 2 cm x 2 cm. The lids were hinged by two rings through both lid and tray on one of the long sides and a stainless steel clip hasp lock on the opposite side. Each tray was bridled by a 6.4 mm braided nylon line from each top corner that were joined about 1 m above the tray with a loop. Another 6.4 mm nylon line was fastened to the loop on one end and to the station on the other. Stations 8, 9 and 10 were suspended, but touching the bottom, from permanent structures; all other trays were set on the bottom with the line attached to either a buoy anchor chain, a ring on a day beacon, or to private docks. All trays had two bricks tied on edge to their bottom to hold the trays above the muck.

Sampling Visits

A total of nine visits were made to the study area. Eight visits were collecting and observation field trips after the stations had been established on 15 May. These visits were made on 23 May, 2 June, 14 June, 27 June, 12 July, 4 August, 23 August and 18 September. Not all stations were visited on each field trip due to mechanical breakdown of the outboard motor. Dates of visits and stations sampled are given below.

Visit 1, 15 May--Was done from R.V. AQUARIUS and established the stations under the guidance of Dr. George Krantz. All subsequent visits were made by outboard runabout.

Visit 2, 23 May--Twelve oysters were collected from every station except station 1 where the tray had been turned upside down with the lid open. Qualitative notes were kept on presence or absence of organisms associated with oyster communities. For hydrographic data see Table 1.

Visit 3, 2 June--Visited stations 2, 3, 4, 8, 9 and 10 successfully. Station 1 had been abandoned. Station 6 could not be found. Stations 5 and 7 had gotten so badly tangled up in the rusty metal of the day markers that the trays could not be brought to the surface for inspection and sample collection.

Visit 4, 14 June--Successfully collected samples from stations 3 - 10.

Visit 5, 27 June--Successfully collected oyster samples from stations 2, 3, 4, 6, 7 and 8. Station 5 had been removed by vandals. Outboard motor trouble prevented sampling of stations 9 and 10.

Visit 6, 12 July--Collections were made from stations 2, 3, 4, 6, 7, 8, 9 and 10.

Visit 7, 4 August--Collected from stations 3, 4, 6, 7, 8, 9 and 10. Station 2 could not be retrieved by either grapnel or by diving. Efforts to locate this station's trays on subsequent visits failed.

Visit 8, 23 August--Failed to bring up station 4 with either grapnel or diving. Samples were taken at stations 3, 6, 7, 9 and 10.

Visit 9, 18 September--Ended the observation period. One tray from each remaining two-tray station was taken out. The remaining trays were left to be collected at a later date so that oysters could be checked for phthalic esters after a full growing season. However, those trays tied to the clam buoys were taken up by D.N.R. and no identification of trays could be made.

Controls--A control station was established on 15 May at the pier at Solomons. Sampling of the controls coincided with every visit to the Chester River stations.

Oyster Scoring Procedures

Oysters were brought into the lab for gross examination and preservation. Oyster samples collected from the tray stations were packed in wet paper towels and kept under refrigeration until they could be examined. Oysters were shucked by severing the aductor muscle at the left valve and displaying the oyster in its right valve with the left valve removed. Features described were shell height, new growth, color of the meats, condition (which will be further explained) and possible imperfections. Most common imperfections were *Polidora websteri* infections, shell ulcers, muscle ulcers, and mud blisters. Tissue ulcers and bill obstructions were rare and no pea crabs *Pinnotheres ostreum* or sponge penetration by *Cliona celata* were found (salinity too low).

Length and new growth measurements were recorded in mm, but imperfections and infestations were scored on a scale of 0 (not present) to 5 (omnipresent). The scoring of observed oyster condition (not to be confused with condition index (CI) which is a totally different procedure) was also according to a scale of 0 to 5 but deserves elucidation of its criteria:

0 = Dead.

1- = An oyster that is completely transparent is definitely unhealthy, all organs clearly visible (no gonad or glycogen). It does not fill its shell cavity but lies like a limp piece of clear gelatin on its valve. This oyster is moribund.

1 = Same as above with slight nuances for improved scoring. This oyster may live.

2 = Oyster not transparent but liver (digestive gland) clearly visible. Gonad or glycogen low and patchy. Does not fill its shell cavity well. Not moribund.

3 = Average looking. Reasonable amount of gonad and/or glycogen. Liver barely visible. Mantle appears to be an active, well-functioning organ. If examined immediately after collection a crystalline style may be found.

4 = Good looking. Enough "fat" to cover liver completely. Plumper than 3, therefore, fills its shell cavity shell. Mantle uniform rich color. Mantle edge active to stimulae. Healthy heartbeat. Solid crystalline style upon examination immediately after collection.

5 = Excellent looking. The oyster appears cramped by its shell cavity. It bulges over the half shell with thick layers of gonad and/or glycogen. Clean uniform coloration throughout. No deficiencies of any kind noted.

Upon completion of this gross examination, the oyster meats were individually wrapped in cheesecloth together with a numbered tag for later identification and preserved in Davidson's fixative.

Data for new growth and mean condition score were compared using Student's t-test.

Possible Point Source Studies

A 96-hour toxicity study was performed at stations in the vicinity of possible point sources of pollution. These sources were the Chestertown Sewage Treatment Plant, the Tenneco chemical plant which manufactures plasticizers and the Campbell's Soup Company where chicken carcasses are prepared and diced. Tenneco and Campbell's discharge into Morgan Creek while the Chestertown Sewage Plant discharges into Radcliffe Creek shortly before it joins the Chester River at the southern edge of Chestertown.

Stations--

There were a total of six stations. The control station was located at least 1-1/2 km upstream from the Tenneco plant. This station was in a small tributary to Morgan Creek but the same one which carries the overflow from the discharge pond of the Tenneco Pond to Morgan Creek.

Tenneco Pond--located in the Tenneco discharge pond about 6 m away from the standpipe which is the point farthest away from the plant's point of discharge. Depth at this station was about 0.6 m.

Tenneco Downstream--in the receiving stream from the Tenneco Pond about 350 m downstream from the pond. Depth at this station was 0.5 m maximum. No tidal influence was noted.

Campbell Upstream--in Morgan Creek near the middle discharge pipe of the Campbell leaching fields in 0.8 m depth at low tide. Due to tidal amplitude, this station could only be checked once a day as at high tide the depth became 1.6 m or more.

Campbell Downstream--also in Morgan Creek about 500 m from station upstream and located just upstream from Campbell's most downstream discharge. Tidal difference here was also more than 0.6 m, which often limited mortality checks to once a day.

Sewage Plant--was located in Radcliffe Creek about 6 m downstream from the sewage outfall. Tidal difference varied depth from 0.5 m to well over 1 m, which allowed only one inspection per day.

Procedures--

Experimental species for this investigation were the golden shiner, Notemigonas crysoleucas and the crayfish, Procambarus acutis acutis. These species were chosen because they are native to the area, are easily available, and easily acclimated to new conditions and represent animals which are neither overly delicate nor particularly pollution resistant forms. Golden shiners were obtained from Green Valley Minnow Farms, Brogue, Pennsylvania. Crayfish were obtained by seining various creeks, ditches, and rivers on the Eastern Shore. All animals were held for a minimum of 2 weeks in sand-filtered Solomons well water. Temperatures were adjusted to those that were expected to be encountered in the field. Both species were fed Purina Trout Chow® daily. Transportation to and from the laboratory was in a 1400 l tank aerated using tanks of compressed air.

Test animals were contained in cages of 30.5 cm x 30.5 cm x 91.4 cm with a 13 mm rebar frame covered with 3.2 mm mesh nylon netting.

The experiment lasted 96 hours. One cage with 30 golden shiners and one cage with 15 crayfish were put overboard at each station. Where possible, the cages were checked twice daily and dead animals were removed and preserved for future autopsy. Once a day the animals were fed Purina Trout Chow®.

All survivors of the 96-hour experimental period were preserved in Davidson's preservative for later autopsy.

Hydrographic data, recorded at every visit, were temperature, salinity, dissolved oxygen and residual chlorine (see Table 2 for these data).

Autopsy of Specimens--

The autopsy of golden shiners was divided into two parts--external and internal features. External features included standard length, examination for damage such as broken, absent of excessive mucus covering; missing or deformed scales, abrasions on body and/or fins; and afflictions such as fungus, discolorations, cysts and parasites. Internal autopsy examined general appearance, color, damage and foreign material on or in the buccal cavity, gills and gill arches; texture, size, color, content and abnormalities of stomach, internal and external intestinal lining, liver, gasbladder, gonads, spleen and adipose tissue. Any observations that did not fit into the prepared autopsy sheet were recorded under item "Other." All specimens were individually wrapped in a bag of cheesecloth containing an identifying tag and were placed in fresh Davidson's solution.

Cages containing crayfish were checked at the same time as the cages with golden shiners. At that time dead crayfish were removed and preserved in Davidson's solution. All surviving crayfish were preserved at the end of the 96-hour experimental period.

Autopsy of crayfish examined both external and internal features. External features such as injury, regeneration to appendages and body, hardness of cephalothorax, color, affliction with fungus, discoloration, and bacterial infection were recorded.

Internal features such as condition and foreign material in the gills and gill chamber, texture and content of cardiac and pyloric stomach, condition and appearance of heart, hepatopancreas and gonads were recorded.

After autopsy the specimens were wrapped individually in a cheesecloth bag that contained an identification tag and placed in fresh Davidson's preservative.

Autopsy data were examined for correlations using contingency analysis with chi-square tests.

TABLE 2. HYDROGRAPHIC OBSERVATIONS FOR THE POSSIBLE POINT SOURCE STUDIES

Site	Sept. Date	Time	Temperature (°C)	Salinity (°/°°)	Dissolved Oxygen (ppt)	Total Residual Chlorine (ppm)
Controls	25	1430				
	26	1400	15.2	0	6.9	0
		1800	15.5	-	10.4	-
	27	0645	12.0	-	6.7	-
		1800	-	-	-	-
	28	0800	13.5	-	6.5	-
		1500	17.2		4.8	
	29	0930	11.3	-	6.8	-
Tenneco Pond	25	1135				
	26	1300	21.0	2	13.8	<0.05
		1900	21.0	2	17.8	-
	27	0730	16.0	0	8.4	-
		1845	25.	<1	20+	0.04
	28	0845	17.5	<1	10.3	0
		1545	21.1	2	16.2	0.01
	29	1200	17.3	-	7.2	<0.02
Tenneco Downstream	25	1100				
		1900	19.5	-	3.8	-
	26	1230	15.0	0	6.8	0
		1930	-	-	-	-
	27	0815	12.5	0	4.8	-
		1930	18.3	-	5.2	-
	28	0930	14.8	-	4.2	-
		1630	18.6	1.7	4.5	-
	29	1230	14.8	0	6.2	0

(continued)

TABLE 2. (continued)

Site	Sept. Date	Time	Temperature (°C)	Salinity (°/oo)	Dissolved Oxygen (ppt)	Total Residual Chlorine (ppm)
Campbell Upstream	25	1036	-	2	-	-
	26	1110	18.0	0	3.4	0
		1630	21.0	1	12.5	<0.01
	27	1015	19.0	<1	10.6	<0.02
		1600	20.3	-	11.7	-
	28	1015	18.9	0	9.9	<0.01
		1330	19.8	<1	13.2	<0.02
	29	1315	13.7	3	13.6	<0.01
Campbell Downstream	25	0945	-	-	-	-
	26	1000	19.3	1+	2.3	<0.05
		1600	21.5	2	9.5	<0.02
	27	1115	19.5	<1	9.8	<0.01
		1700	21.0	2	8.4	<0.04
	28	1100	18.6	<1	9.3	<0.01
		1415	20.2	2	9.8	0
	29	1330	19.2	3	12.5	0.01
Sewage Plant	25	1510	-	2	-	-
		1800	19.0	-	4	-
	26	1500	19.3	2.5	6.5	0.45
	27	1300	18.5	2	5.8	<0.02
	28	1230	17.8	1	5.8	<0.01
	29	1430	17.5	4	8.7	0.12

RESULTS

Oyster Experiments

Mortality over the period was low (see Table 3). The only station showing mortality noticeably higher than that of the control station is Station 10. This is the most upriver station and subjectively seemed initially to have lower concentrations of fouling organisms and more silt. The single event of six dying, between 2 June and 14 June, may be related to heavy runoff and smothering by silt.

TABLE 3. MORTALITY OF OYSTERS DURING THIS STUDY

	Station									
	2	3	4	5	6	7	8	9	10	Control
23 May	0	0	0	*	0	*	0	0	0	2
2 June	1	0	0	*	*	*	1	0	1	1
14 June	*	0	0	0	0	0	1	0	6	0
27 June	0	1	0	1†	1	0	0	*	*	0
12 July	*	0	0		0	1	0‡	0	0	0
5 August	*	0	1		1	1	1	1	1	0
Total	1	1	1		2	2	3	1	8	3

* Trays were not examined.

† Lines cut and tray missing.

‡ Tampered with but appeared intact

In late spring and early summer, growth was mostly not significantly different from that shown at the control station, and there was no pattern of either increased or decreased growth (see Table 4). In July, all stations showed significantly slower growth than the controls while in August four out of seven stations showed significantly slower growth.

TABLE 4. COMPARISON OF MEAN NEW GROWTH IN OYSTERS PLANTED IN CHESTER RIVER*

	Station									
	2	3	4	5	6	7	8	9	10	Control
23 May	6.25 (N.S.)	5.88 (N.S.)	5.75 (N.S.)		3.10 (0.001)		4.29 (0.01)	5.08 (N.S.)	5.82 (N.S.)	6.10
2 June	3.75 (N.S.)	4.20 (N.S.)	4.67 (0.02)				4.17 (N.S.)	3.92 (N.S.)	3.73 (N.S.)	2.83
14 June		4.33 (N.S.)	3.50 (N.S.)	5.50 (N.S.)	4.08 (N.S.)	5.92 (0.01)	4.58 (N.S.)	3.15 (N.S.)	4.42 (N.S.)	4.33
27 June	3.17 (N.S.)	4.00 (N.S.)	6.00 (N.S.)		4.67 (N.S.)	6.92 (N.S.)	5.50 (N.S.)			6.00
12 July	4.50 (0.001)	5.83 (0.01)			4.33 (0.001)	6.04 (0.01)	5.67 (0.05)	5.50 (0.001)	3.25 (0.001)	8.17
5 August	4.20 (0.001)	9.08 (N.S.)			7.92 (N.S.)	6.17 (0.01)	6.83 (0.02)	7.42 (N.S.)	1.42 (0.001)	8.44

* Means are expressed in mm. Numbers in parentheses are probabilities that these means do not differ significantly from control means. N.S. indicates probability values larger than 0.05.

There were few significant differences from the controls in observed condition scores (see Table 5), however there is a pattern that lower salinity stations accounted for most of these differences. In all cases but two, where significant differences occurred, the average condition scores were higher than that of the controls. These aberrant stations are Stations 9 and 10 which are the two most upriver stations.

Condition scores are affected by the seasonal condition changes that normally occur in oysters and only partially reflect (except in extremes) the oyster's health. Condition scores are based on the amount of solid color and how well the oyster fills its shell. The buildup of gonad in the spring and the accumulation of glycogen in the fall tend to elevate the score. Conversely, low scores should be expected at the end of summer when oysters are completely spawned out and have suffered through unfavorable high summer temperatures that adversely affected their pumping and feeding. Also, oysters that have come through a hard long winter and spring with low temperatures and low available food have used up a great deal of their reserves (glycogen) and therefore will score low. Still the health of these oysters would not be as different as the two separate scores seem to indicate. Salinity can also influence oyster condition. Oysters in low salinity upriver areas that often warm up early will start their gonad development earlier than oysters in the more saline downriver or bay areas that warm up slower. Freshets often cause upriver salinities that are too low for spawning at spawning temperature. These oysters will not spawn and in the fall convert their gonad material immediately into glycogen without going through a "summer slump." These oysters may score high all year round. However, during the same period the oysters downriver are likely to spawn because the salinity is favorable at spawning temperatures and enter the fall in low condition. After temperatures drop (usually later than upstream), they will feed effectively again and increase their score until the water temperature is 5°C when they cease feeding and rely on their reserve food which decreases their score. Thus, oyster scores should be evaluated with season and location in mind using as many individuals as possible. It is a subjective comparative judgment and should be performed as much as possible by one person to eliminate differences between workers.

Associated organisms and time of occurrence are given in Table 6. When trays were inspected on 12 July, there were no live commensal organisms present at Station 9, and at Station 10 the shells were almost clean except for a few live barnacles, bryozoan colonies and amphipods. During inspection of oysters on 5 August, it was noted that very few commensal organisms were present at Stations 6, 7, 8, 9 and 10 and that the shells were cleaner than before. On 23 August, it was noted that algae and barnacles were dying and decomposing at Stations 6 and 7, and

TABLE 5. COMPARISON OF MEAN OBSERVED CONDITION SCORES IN OYSTERS PLANTED IN CHESTER RIVER*

	Station									
	2	3	4		6		8	9	10	Control
23 May	3.33 (N.S.)	3.33 (N.S.)	3.01 (N.S.)		3.07 (N.S.)		3.11 (N.S.)	3.25 (N.S.)	3.46 (N.S.)	3.31
2 June	3.18 (N.S.)	3.20 (N.S.)	3.68 (0.05)				3.38 (N.S.)	3.23 (N.S.)	3.18 (N.S.)	3.28
14 June			3.79 (N.S.)	3.68 (N.S.)	3.65 (N.S.)	4.34 (0.001)	4.33 (0.001)	3.85 (N.S.)	+2.99 (0.01)	3.46
27 June	3.55 (N.S.)	3.72 (N.S.)	3.61 (N.S.)		3.74 (N.S.)	3.83 (N.S.)	4.27 (0.01)			3.56
12 July		3.58 (N.S.)	3.60 (N.S.)		3.73 (N.S.)	3.63 (N.S.)	4.78 (0.01)	+3.21 (0.05)	3.95 (N.S.)	3.74
5 August		4.06 (N.S.)	3.42 (N.S.)		3.67 (N.S.)	4.74 (0.001)	4.75 (0.001)	4.83 (0.001)	4.68 (0.001)	3.49

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* Numbers in parentheses are probabilities that these means do not differ significantly from control means. N.S. indicates probability values larger than 0.05.

+ Values significantly lower than control value.

TABLE 6. ASSOCIATED ORGANISMS BY STATION

	Station				
Organisms	2	3	4	5	6
Filamentous green algae	1*	2,3,4			3,7
Sea lettuce		1,4			3,7
Brown algae					
Bryozoans			3,6,8	3	4,5,6,8
Hydroids					
Nereid polychaetes		6,7	6		6
Mussels (*Brachyodontis recurvis*)		5,6	6,8		4,5,6,7
Gammarid amphipods	2	1,2	2,4,5	3	4,7
Corophid amphipods		4,5,6,8	3,6,8		5,6
Grass shrimp (*Palaeomonetes* sp.)				3	4,5,6
Xanthid crab (*Rhithropanopeus harrisii*)		5,6,7,8	4,5,6	3	4,5,6,8
Blue crab (*Callinectes sapidus*)		5,6			4,5
Barnacles	4	3,4,5,6,7,8	3,4,6	3	3,6,7,8
Naked goby (*Gobiosoma bosci*)			6		

(continued)

TABLE 6. (continued)

Organisms	Station			
	7	8	9	10
Filamentous green algae				
Sea lettuce				
Brown algae	3,7	3,4		
Bryozoans	3,5,8	4,5,6,8	3	3,5,6,8
Hydroids	4	4		
Nereid polychaetes	3,5		7	
Mussels (*Brachyodontis recurvis*)	6,7,8	6,7,8	7,8	7,8
Gammarid amphipods	4,5,7	3,4,7	2,3,7,8	2,3,5,8
Corophid amphipods	6	6	6	
Grass shrimp (*Palaeomonetes* sp.)		3,5		
Xanthid crab (*Rhithropanopeus harrisii*)	3,5,6,8	1,2,3,4,5,6,7	1,2,3,6,7,8	2,6,7,8
Blue crab (*Callinectes sapidus*)				
Barnacles	3,5,6,7,8	5,6,7,8	2,6,7,8	5,6,8
Naked goby (*Gobiosoma bosci*)	6			

(continued)

TABLE 6. (continued)

	Comments
Station 2.	Very few associated organisms throughout study; lost, not sampled after 27 June.
Station 3.	Very badly fouled with algae at inspections 1-4; inspected on all dates.
Station 4.	Inspected on all dates.
Station 5.	Not inspected on trips 1 and 2 because of unfavorable tidal currents, lost to vandals after inspection trip 3.
Station 6.	Noticeably less fouled at inspection 6, barnacles and algae dying and decomposing at inspection 7.
Station 7.	Not inspected on trips 1 and 2 because of unfavorable tidal currents; noticeably less fouled at inspections 6 and 7, at insepction 7 algae dying and decomposing.
Station 8.	Noticeably less fouled at inspections 6 and 7.
Station 9.	At inspection 5 the shells were clean, few fouling organisms at inspections 6 and 7, recovered in density at inspection 8; not inspected on trip 4.
Station 10.	Almost clean shells at inspections 5, 6, 7; fouling community dense at inspection 8; not inspected on trip 4.

* Inspection trips

1 = 23 May
2 = 2 June
3 = 14 June
4 = 27 June
5 = 12 July
6 = 5 August
7 = 23 August
8 = 18 September

and fouling communities were still sparse at Stations 6, 7, 8, 9 and 10. By 18 September fouling communities seemed back to normal at all stations inspected; that is, all stations but 2 and 5.

Golden Shiner Experiment

Mortality was high at all stations including the controls. Figure 3 is a graphic representation of mortality over time. Excess mortality, that is mortality of controls minus mortality of experiments, is shown in Figure 4. Ranked in descending order of total survivorship the stations are Tenneco Downstream and Control, with Tenneco Pond, Campbell Upstream, and Campbell Downstream all having the same survivorship and with The Sewage Plant having the lowest survivorship. Sewage Plant station is unique in the excess mortality curves in that all nonzero values are negative, that is mortality was higher than control mortality at all times.

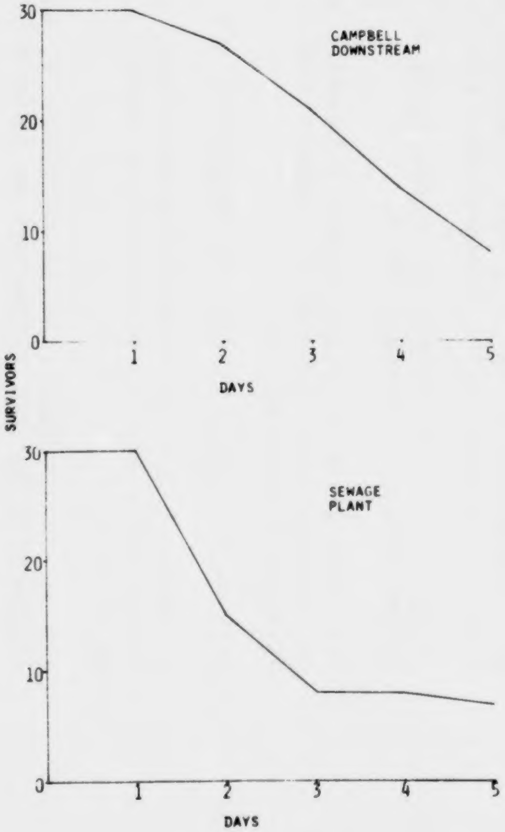

Figure 3. Mortality of golden shiners, by station, over the 96-hour observation period.

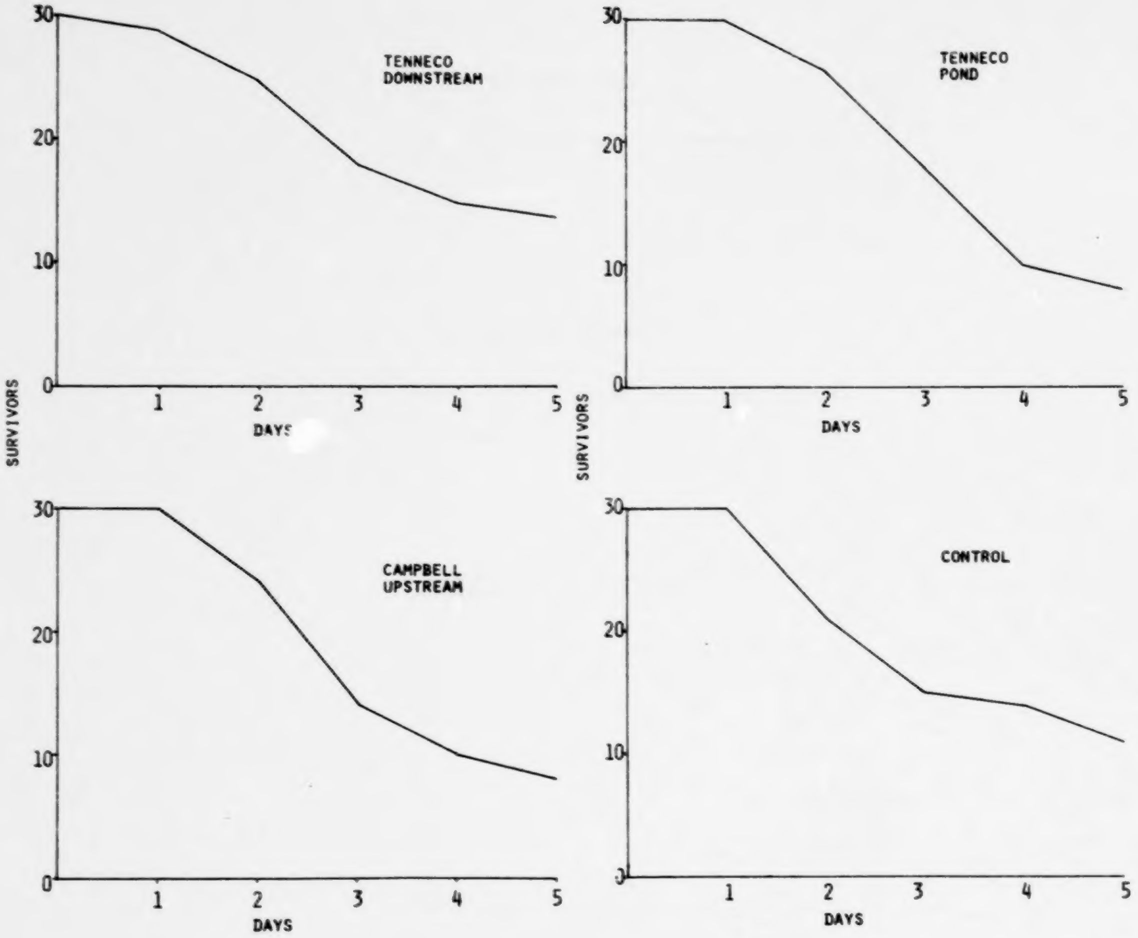

Figure 3. (Continued)

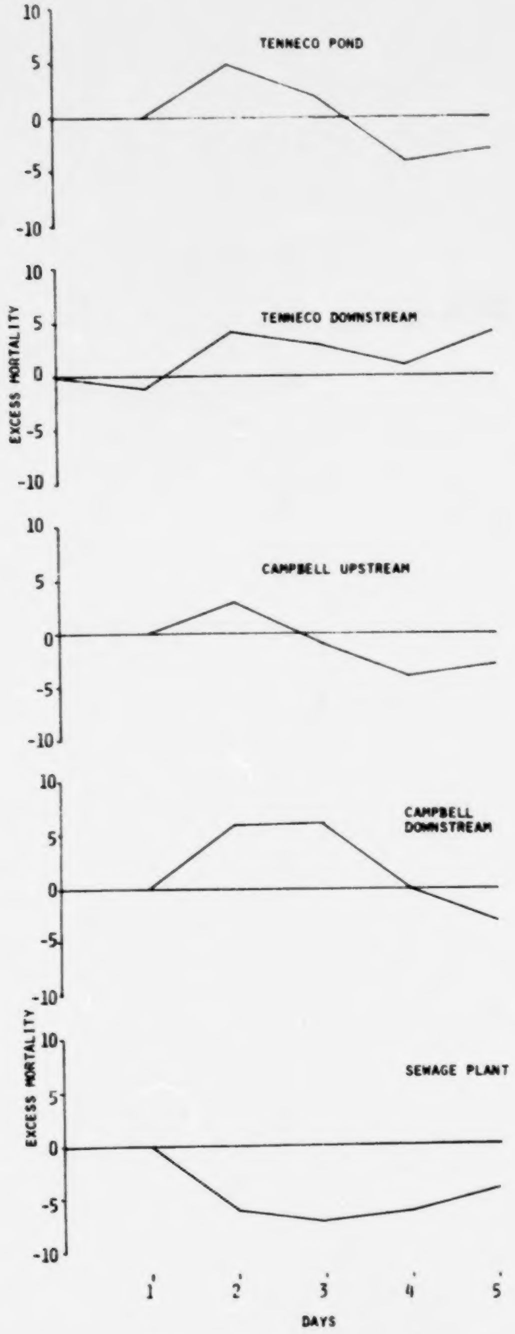

Figure 4. Excess mortality of golden shiners, by station, during 96 hours of observation. This is calculated by subtracting mortality of experimentals from that of the controls. Negative values indicate mortality more than that of the control.

Autopsy data showed the following to be significantly correlated with death at one or more stations (see Table 7 for a presentation of these data); fungus on body or fins; watery fat in the mesentery and on the surface of the intestines; foreign material on the gill surface; and hard, white crystalline nodules attached to the body or fins. Comparing stations for frequency of occurrence of each of these characteristics using nonsurvivors only showed that no location had significantly higher or lower frequency of watery mesenteric fat; Campbell Upstream and Campbell Downstream had significantly higher frequency of occurrence of fungus; the Control Station showed a significantly higher frequency of foreign material (apparently silt) on the gills and Sewage plant, Tenneco Pond and Campbell Downstream showed significantly higher frequency of white nodules. See Table 8 for levels of significance of these characteristics.

TABLE 7. RELATIONSHIP OF NONSURVIVAL TO AUTOPSY DATA*

Station	Fungus	White nodules	Foreign material on gills	Watery mesenteric fat
Control	N.C. †	N.C. †	0.05	0.05
Campbell Upstream	0.005	N.C. †	N.S.	N.S.
Campbell Downstream	0.005	N.S.	N.S.	N.S.
Tenneco Pond	N.S.	N.S.	0.05	N.S.
Tenneco Downstream	0.05	N.S.	N.S.	N.S.
Sewage Plant	N.S.	0.01	N.S.	0.01

* Numbers are probability values calculated using contingency analysis. N.S. indicates that probability was greater than 0.5.

† Not calculable because of zero expected values.

TABLE 8. COMPARISONS BETWEEN STATIONS FOR CHARACTERS RELATED TO NONSURVIVAL*

	Control	Campbell Upstream	Campbell Downstream	Tenneco Pond	Tenneco Downstream
		Fungus			
Campbell Upstream	0.005				
Campbell Downstream	0.005	N.S.			
Tenneco Pond	N.S.	0.005	0.005		
Tenneco Downstream	0.025	0.025	0.005	N.S.	
Sewage Plant	N.S.	0.005	0.005	N.S.	N.S.
		Foreign material on gills			
Campbell Upstream	0.05				
Campbell Downstream	0.005	N.S.			
Tenneco Pond	0.005	N.S.	N.S.		
Tenneco Downstream	0.005	N.S.	N.S.	N.S.	
Sewage Plant	0.005	0.05	N.S.	N.S.	N.S.
		White nodules			
Campbell Upstream	N.S.				
Campbell Downstream	0.025	0.025			
Tenneco Pond	0.005	0.005	N.S.		
Tenneco Downstream	N.S.	N.S.	N.S.	N.S.	N.S.
Sewage Plant	0.005	0.005	0.005	0.025	0.005
		Watery mesenteric fat			
Campbell Upstream	N.S.				
Campbell Downstream	N.S.	N.S.			
Tenneco Pond	N.S.	N.S.	N.S.		
Tenneco Downstream	N.S.	N.S.	N.S.	N.S.	
Sewage Plant	N.S.	N.S.	N.S.	N.S.	N.S.

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* Numbers are probability values calculated using contingency analysis. N.S. indicates that probability was greater than 0.05.

Crayfish Experiments

No significant mortality was seen at any station, and autopsies failed to disclose any obvious difference that could be related to treatment between the crayfish that died during the experiments and those that survived.

DISCUSSION

Oyster Experiments

Since one of the principal objectives of these studies was to identify possible causes of oyster mortality in the Chester River since 1974, it is unfortunate for the study that no large-scale mortality occurred at any of our stations. Observations by scientists, watermen and representatives of the Department of Natural Resources have noted that oysters that die in the "mortality areas" of the Chester River have unusually clean shells showing little or no fouling. We did note a dieoff of fouling organisms. This dieoff was first noted at the two most upriver stations on 12 July and coincided with a fishkill in the river. Our personnel noted that the fishkill was a widespread phenomenon with dead fish, mostly catfish and carp occurring from near the mouth of the river to well above the mouth of Morgan Creek. It might be speculated that the two phenomena are related in some manner.

The dieoff of fouling organisms was never detected downstream below Station 6 (clamline buoy A off Corsica Neck) and seeming full recovery was noted by 18 September. An examination of Table 3 will show an interesting pattern of deaths. If we ignore the unusual high mortality at Station 10 on 14 June, which may be related to high runoff and silt, 6 of the 13 remaining mortalities occurred during the period of the fouling organism dieoff and at the stations where commensal organism dieoff was noted. It may be that the oyster dieoff did occur during the study, but for some reason this was an unusually mild year for it.

An examination of Table 4 shows that summer growth rates of our oysters placed in the Chester River were significantly lower than that of the control oysters held in the Patuxent River. This reduced growth rate coincides with the period of time when the dieoff of fouling organisms occurred.

Table 5 shows that oysters in the four most upriver stations had significantly higher observed conditions scores. These may well relate to salinities being too low for spawning. These oysters may simply have never lost gonadal material or glycogen.

One possible hypothesis for the dieoff has been suggested by Donald Heinle (personal communication). He suggests that heavy loading of the system with organic material will cause the water to go anoxic or nearly anoxic at depth, and under such conditions carbonic acid is produced which will dissolve the outer layer of the oyster shell; thus, producing the "scrubbed clean" look of the oysters. He has seen this occur in estuarine waters and in the laboratory despite the heavy buffering of the salts present. If in addition to producing carbonic acid, anoxic conditions hold for a long period when the oysters were otherwise stressed, then mortality would occur. This hypothesis is consistent with the data and cannot be disproved as our sampling periods were far enough apart that we might not have detected the event causing the anoxia.

Golden Shiner Experiments

Table 7 indicates that different stations show different correlations between nonsurvivorship and four factors noted during autopsies. Both Campbell stations and Tenneco Downstream had significantly higher infection percentages of fungus. The fungus appeared to be *Saprolegnia* sp. but was not identified. *Saprolengia* is not an obligate parasite. Usually it occurs as a saprophyte. Its abundance at these stations indicates high concentrations of organic material.

The white nodules are problematic. Their identification is unknown. They are white, translucent, hard, brittle and subspherical to ovoidal to rounded irregular in shape. Largest diameter is less than 1.5 mm. They occur in patches usually on the ventral half of the body or on the fins. They are firmly attached to the scales, fin rays or opercle but can be broken loose by scraping. None were noted in specimens which had survived nor in any specimen from Campbell Upstream or the control station. It was thought that these might be the results of an interaction between the calcium of the boney scales and fin rays and some ingredient in Davidson's fixative. Arguments against this are numerous. The crayfish have as much or more calcium in their exoskeletons than the fish scales, but no crayfish was ever found with one. The placement on the body in discrete patches argues against any random process, and the fact that there were significantly more or less of these at certain stations adds doubt to any simple explanation.

Foreign matter on the gills irritate the gills, cause stress symptoms and synergistically increase toxicity of other substances.* It is therefore conceivable that the foreign material noted on the gills of fishes that died at the control station was a contributing factor to their death. In addition to silt particles on the gills, fibers resembling those from toilet paper were noted on one fish's gills. This indicates that untreated domestic sewage may find its way into the stream above our control station.

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* Wilber, Charles G. 1976. The biological aspects of water pollution. Charles C. Thomas, Publisher, Springfield, IL, 296 p.

What we have referred to as "watery fat" is a type of fatty tissue that has a flabby look and a semiliquid consistency. It appears under low magnification to be translucent and to have inclusions of water or other liquid free in it. This fat was found in both surviving and dead specimens but a significant correlation between it and the nonsurvivorship was noted at the control station and at the sewage plant station. No mention of this kind of fat in the literature was found, but it was speculated that the presence of watery fat may be an early symptom of stress. In prolonged periods of high energy expenditure and during starvation, body fat is mobilized to act as an energy source. If lipids are used up, fat cells may lose their turgor producing this soft,gelatinous texture.

The fact that dead fish from different stations have very different patterns of correlations with these factors indicates that there are probably multiple causes of mortality and that each station has its own unique combination of factors. In short, no single cause of mortality was found nor was it thought that the mortality noted in these experiments is necessarily related to the cause of oyster mortality downstream.

Crayfish Experiments

Mortality was too low and autopsy data failed to find any changes which could be related to treatments. It seems that this species of crayfish is hardier than the golden shiners. The mortalities occurred mostly with crayfish that had moulted and that were killed then by other crayfish.

SECTION 5

PHTHALATE ESTERS AND RELATED CHEMICALS IN THE CHESTER RIVER

INTRODUCTION

The immediate purpose of this work is to assess the potential for bioaccumulation of alkyl phthalates as a way to explain the recent past mortality of oysters in the Chester River. Our starting approach involved dual plans to use both gas and liquid chromatography. Careful technique is needed to avoid contamination by the ubiquitous presence of the phthalate esters in the environment, including their reported presence in the chemical laboratory. Our own facilities proved to be no exception.

Two main problems needed to be solved: positive errors due to contamination during the measurement process, and negative errors due to incomplete extraction. Hopefully, both problems have been solved. Several models were considered for influx of alkyl phthalate waste from the Tenneco plant. These considerations were used to guide the sample selection and interpretation process. In brief, our overall approach emphasized measurement reliability, it narrowed the number of samples for analysis, and it gave a conceptual base to guide future work. Alkyl phthalate contamination in the Chester River clearly includes di(2-ethylhexyl)phthalate (DEHP). Our finding of massive contamination in Tenneco Pond is consistent with the pollution consequences that are consistent with the existing discharge permit which has been approved by the State of Maryland.

Geographic and Background History

The Tenneco factory is sited on a tributary of Morgan Creek approximately 7 miles (10.6 km) upstream from where the creek empties into the Chester River. The location is shown in Figure 5. The oyster mortality starts 12 miles (19.2 km) downstream from Morgan Creek toward the mouth of the Chester River. The mortality involved a downstream progression. The progressive movement is evident in Figure 6, which shows the status of mortalitities in oyster beds monitored in 1970-1975 (see Appendix A). The progression of the total mortality zone (0 percent live oysters) is seen by comparing the 1973 and 1975 results where the mortality front is seen to advance 5.2 km in the downstream direction.

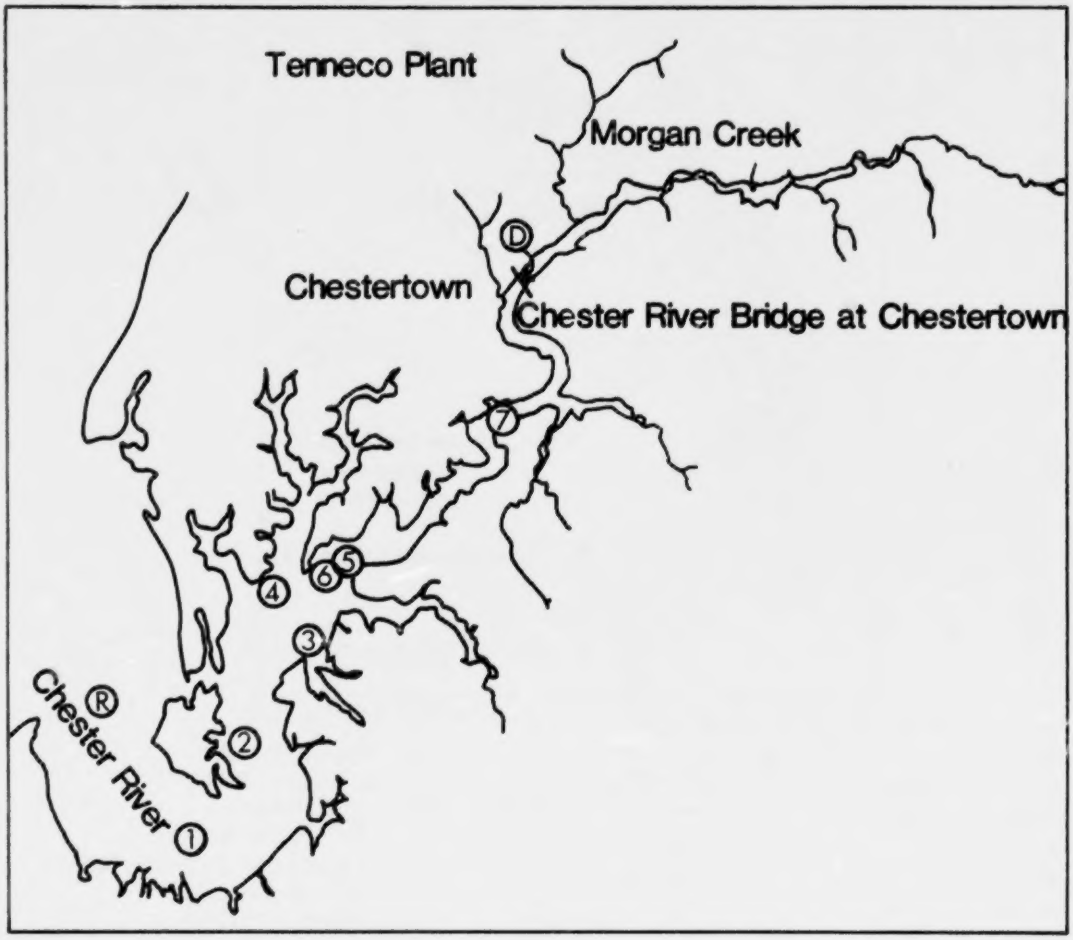

Figure 5. Map of Chester River illustrating sample sites. Site coordinates are given in Table 18.

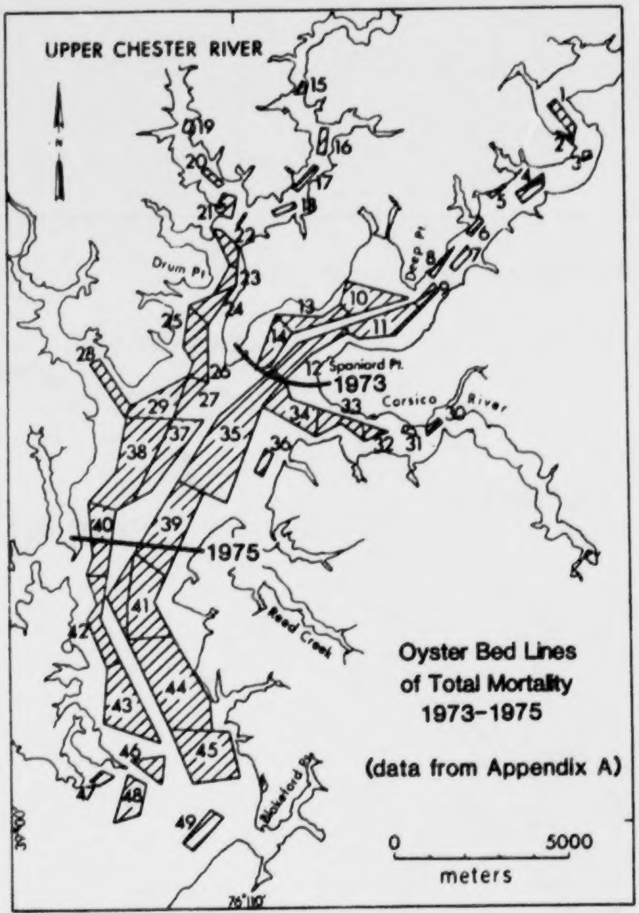

Figure 6. Downstream progression of oyster mortality.

1. Northwest
2. Melton Point
3. Booker Wharf
4. Hollyday
5. Haddaway
6. Shippen Creek
7. Mummy's Cove
8. Deep Point
9. Sheep
10. Commegy's Bight
11. Emmory Hollow
12. Spaniard Point
13. Cliff
14. Ebb Point
15. Ware
16. Phillip
17. Bailey
18. King's Creek
19. Wilson's Point
20. Eagle Point
21. Island Point
22. Davis Creek
23. Drum Point
24. Boathouse
25. Sand Thistle
26. Hudson
27. Nichols
28. Limekiln
29. Willow Bottom
30. Possum Point
31. Ship Point
32. Emory Wharf
33. Town Point
34. Holton Point
35. Oldfield
36. Robin's Cove
37. Chester River Middleground
38. Bluff Point
39. Hell's Delight
40. Bay Bush Point
41. Piney Point
42. Belts
43. Durdin
44. Horse Race
45. Carpenter Island
46. Black Buoy
47. Hail Creek
48. Hail Point
49. Poplar

(From: Meritt, D. W. 1977, Univ. Md. Ctr. Environ. Estuarine Studies Spec. Rpt. 7.)

The discharge of any toxic chemical might account for the mortality pattern. The Tenneco plant waste discharge is one possibility based on circumstances that will be considered here.

The Tenneco plant became operational in 1959. Esterification equipment was acquired in 1965. A process for easte treatment by bacterial digestion was installed in 1968. At this time, a dam was located across the water flowing from the Tenneco plant. As a result, a small pond (Tenneco Pond) was formed with 40 x 10^3 m^2 (10 acres or 4 hectares) of surface area.

Hurricane Agnes occurred in May of 1972 bringing 16.0 cm of rainfall in one day (Palmer 1972) to the plant area which was flooded. The contents of a waste collection tank were washed out. It was estimated that 50-100 gallons (190-380 L) of organic waste liquid were washed out into Morgan Creek and then into the Chester River. It is possible that the washed-our chemicals could be causally related to the oyster mortality. (It is also possible that the effect might be the result of one or more other factors, including the continual discharge of unidentified organic compounds which reside after waste treatment, agricultural runoff, a "natural phenomenon," etc.). Various pathways for the transport of phthalate esters from the Tenneco plant to the oyster mortality area are now discussed.

The Upper Bay Survey (Palmer, Schubel, and Cronin 1975) makes it clear that sparingly soluble organic compounds are specifically associated with the finely has been established with observations that the presence of such organic compounds is precisely correlated by the octanol-water partitioning model, and the adsorption of neutral organic compounds by sediment has been strongly correlated to the weight fraction of organic carbon (Karicknoff et al. 1979). This finding is consistent with the known tendency of humic acid to adsorb such compounds rather tenaciously. Schnitzer's work (1972) suggests possibly irreversible adsorption of phthalate esters by clay soils, although there is some controversy on the significance of that finding. Regardless, the known distribution of neutral organic compounds in the marine ecosystem includes biomagnification by life in the marine environment, and a corresponding adsorptive magnification effect into the natural clay-organic complexes present in marine sediments. Since dioctylphthalate (DOP) was observed early in the study as a conspicuous and major component in Tenneco Pond sediment, it is important to show whether or how that material would be transported from the Tenneco factory to the clay-rich sediments in the Chester River. These latter sediments according to the Chester River Report (Palmer 1972) occupy the deeper terrace portions of the river floor and the transitional channels between nearshore and the main river channel. Taken in perspective, these are the most critical samples where investigation can be focused. Such sediments are widely recognized for their ability to adsorb compounds for long periods of time, geological epoch in many instances. As a result, the clay-humic structure contains a historical reservoir of many compounds deposited through natural or anthropogenic influx. In the present study where no biological specimens were preserved, the key to the retrospective analysis of the past oyster mortality would have to told by the sediment composition.

DISTRIBUTION PATTERNS

Three factors which may modify the distribution patterns of organic distribution in the Chester River as as follows.

a. The downstream dilution effect. The width of the Chester River grows from roughly 0.5 km at Chestertown to 1.6 km near the midway region close to Spaniard Point to 5 km at the mouth. This suggests that the sediments, behaving as a pollutant sink, may become more dilute in the pollutant as one proceeds downstream. In the initial pollution history of a tidal river, this would would be more likely than later on when the accumulative pollution may build toward a uniform pollutant concentration.

b. Uniform recirculation model. The Chester River, as a tributary estuary, is moderately stratified into two layers as described by Pritchard (1967). The net advective surface water flow is in the upstream direction while that in deeper water is downstream. Suspended or dissolved matter is dispersed in both directions as a result of two processes: (1) diffusion of the dissolved solute across the thermocline so that it is spread into layers whose net motions are in opposite directions. It is likely that the nonpolar plasticizers will quickly (2) sorb onto suspended sediment and then settle down to the leptopellic layer on the sediment surface, and then either deposit or become resuspended into a circulation cycle. These circula-processes may cause wide mixing, motably upstream as well as downstream.

c. "Hot spots." As noted in Han's dye tracer experiments (1972), a pollution plume is apt to remain intact during the first few tidal cycles. That pattern was shown to exhibit selective localization effects. This would produce the opportunity for hot spots where an elebated concentration of pollutant may deviate significantly from the model based on complete mixing.

PROBLEM STATEMENT

It seems fundamental that environmental influx of toxic pollutants is feasible for study when the pollutant is identified, or at least limited to be among a group of candidates. In the present study, this line of first approach was blocked from the beginning because none of the affected oyster tissue had been saved. The circumstances are such that the toxic agent (if there was any) may have been introduced, reached toxic levels, and returned afterwards to pretoxic conditions. Although alkyl phthalates are readily degraded by bacteria, photolysis, and hydrolysis, it is our belief that the known persistence of alkyl phthalates in the marine environment is the result of rapid sorption by suspended clay-rich matter which eventually settles through sedimentation. Once sorbed, the rate of degradation is indefinite, but it is slow enough that the phthalates are considered "persistent." In view of the rapid bioturbation process, surficial sediments (upper 10 cm) are quite likely to carry memory effects due to accumulative pollution during the previous decade.

The difficult part in the present study was to develop reliable methods of measurement since it was felt that none of the initially available measurement technology was reliable enough or accurate enough for the purposes of this study.

The principal focus in the work to be presented next is based on the significance of the clay-rich sediment regions below a 2-8 m water column. These should contain the residual compounds that may help to understand whether threatening or tpxoc plasticizer concentrations exist now, or were likely within the past decade. Since sedimentation occurs at an estimated rate of 1 cm per year, our efforts were focused on values in the top 10 cm.

Based on preliminary studies we initially picked sampling sites included apex of the mortality. The sites were chosen as likely to show whether or not the alkyl phthalates could have caused the oyster mortality. The first region is Tenneco Pond where the sediment composition was found to be highly polluted. The second region involves a series of five samples taken at the apex of the oyster mortality. That is where the downstream mortality movement was not clearly in evidence. The third region is at the mouth of the Chester River. That region is likely to contain a representative mixture of pollutants from the upper Chesapeake Bay. Since this region is a reference point for possible upstream river pollution, we used a homogenized sample of the river mouth sediment "R" for reference purposes and for independent analysis.

Efforts were also made toward development of methods for analysis of oyster tissue. We underestimated the problems here so that aspect of the study was the last to be completed. These results will be presented later in this report.

EXPERIMENTAL PROCEDURES

Trace Analysis Techniques

The key goals in trace organic analysis of sediment are to achieve complete extraction, to avoid loss, to prevent contami- and to avoid interferences. In preliminary experiments we found that ultrasonic extraction of dry Tenneco Pond sediment with methanol gave easily measured quantities of DOP. Later, tetrahydrofuran was found to give a higher yield. Then, a series of extracting solvents was tested comparatively and dichloromethane was found to give the highest extractive efficiency and relatively short times were required. The duration of the ultrasonic agitation was varied until it became certain that longer extraction periods did not give greater extractive yield.

The true extractive efficiency is not subject to direct measurement. Spiked addition of analyte produces a different sorbed state than the analyte in the sample matrix. To assess the apparent accuracy, two samples were selected for independent measurement as will be described. The approach was to do what could be done to show that re-extraction by baried methods did not lead to a higher yield.

A major problem of contamination arises when measuring traces of plasticizers. The environment is widely contaminated by these substances, and the chemical laboratory is no exception. Their presence has been reported in bottle cap liners, solvents, extraction thimbles, preconcentrating resins and adsorbents, aluminum foil, glass, wood, air and pipet fillers (de Zeeuw 1975, Singmaster et al. 1976, Webster and Nickless 1976).

It is obvious that the reduced contamination can be achieved by deliberately minimizing the overall exposure to the sources of contamination by minimizing solvent volume, the number of manipulations, avoiding contact with any plastic materials, and ultracleaning of apparatus. This can be summarized in two statements: the sampling and processing apparatus must be uncontaminated, and the amount of methodology must be kept to an essential minimum.

Although these aspects led us away from available methodology (Giam et al. 1976), the end of the study showed that our method did come close to similar techniques by Dr. William Budde (EPA-Cincinnati). The procedure used by Budde (ultrasonication, GCMS-selected ion monitoring) differs mainly in the technique used for drying the sample--direct addition of Na_2SO_4 to partly dried sediment. Our method uses the same desiccant but the drying efficiency is greater. Regardless, the two approaches are appropriate for interlaboratory comparison.

The measurement approach is based on a reported (Watson 1976) application of GCMS where the MS section is used in the selected ion monitoring mode (SIM). This provides an impressive gain in selectivity since only the predominant fragment ion masses are monitored. As a result there is a corresponding boost in instrumental sensitivity. The technique uses instrumental detection selectivity to replace the lesser certainty of multiple extractive and separation conditions (Giam et al. 1976).

The basis for the analytical methodology can be seen in the following way. The upper curve in Figure 7 is a gas chromatogram of a dichloromethane extract of a dried sediment sample from the Chester River mouth. This is the working standard, sample "R," located in Figure 5. The omission of any cleanup procedure creates an unresolved bunching of chromatographic peaks due to the many thousands of compounds that are present in the extract. However, when the mass spectrometer is used as GC analyzer, an extraordinary boost to the selectivity of the combined instrumentation is realized. This is shown for mass 149 as the lower curve in Figure 7. The integration of m/z 149 intensity to measure alkyl phthalates was confirmed by comparing integrated area ratios for confirmational fragment ion masses for DBP (m/z 2-5,223) and for DEHP (m/z 167,279). This was done for confirmational purposes to show that no interferences were present at the levels being investigated.

It has been concluded that there is no ambiguity in the present dual use of multiple ion monitoring to identify and measure DBP and DEHP in a single GCMS-SEM experiment. This is a result of the extreme molecular selectivity, and correspondingly justified methodological simplication. The generic term DOP (dioctylphthalate) will be used to refer to one or both of the isomers, DNOP (di-n-octylphthalate) and DEHP (di-2-ethylhexylphthalte). Distinction between these substances was obtained later in the study (see Figure 14).

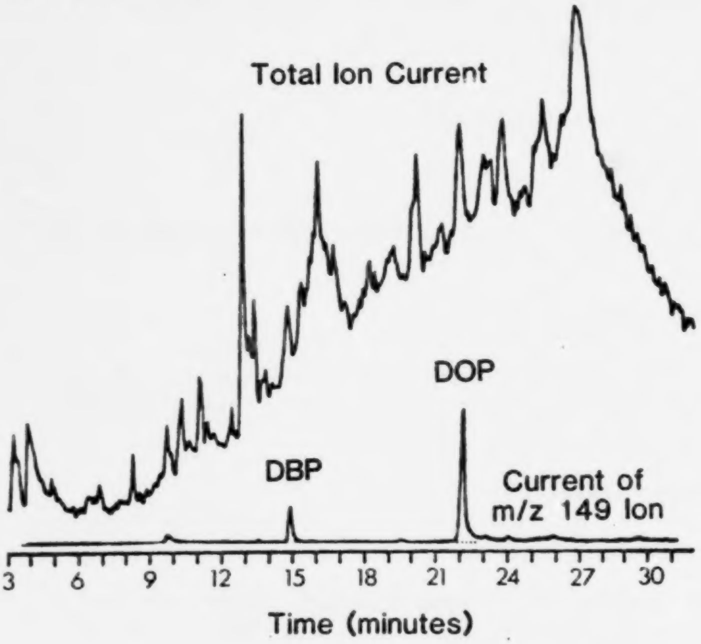

Figure 7. GCMS of Chester River mouth sediment extract.

Cleaning Procedure

Scrupulous cleaning procedures were used to minimize organic contamination of the material used in this work. The details are given in the following.

Glassware (Sample bottles, vials, lab glassware, all glass materials)

1. Wash with Aquanox detergent.
2. Rinse with tap water followed by filtered, distilled water.
3. Bring to annealing temperature of the glass (450°C) for at least 1 hour.
4. Slowly cool (>4 hours).
5. Cover exposed areas with baked aluminum foil.
6. Cover with clean caps or lids.

Tools (Forceps, trowels, spatulas, etc.)

1. Clean with Aquanox.
2. Rinse with charcoal filtered water.
3. Dry in air.
4. Wrap in aluminum foil.

Piston Core Liner (Brass)

1. Scrub with Aquanox detergent using brush fixed on ram-rod.
2. Rinse with tap water.
3. Add chromic acid/sulfuric acid cleaning solution to etch surfaces: tube is inverted several times.
4. Rinse with tap water.
5. Inspect for bright shiny inner surface.
6. In field, tube was rinsed by brush and rinsed between cores using the on-board water supply.

Bottle cap liners (Teflon)

1. These liners were cut from Teflon sheet using cork bores. They were then extracted for 24 hours using Soxhlet extraction.

2. Liners were stored in an air-tight, cleaned glass bottle with an aluminum foil liner.

Solvents

1. Water was purified by passing in succession through activated carbon filter, XAD-2 column (Chesler et al. 1976) and final ultrafilter.

2. All other solvents--dichloromethane, MeOH, etc.--were distilled in all-glass distillation apparatus. Tetrahydrofuran was distilled from freshly cut sodium, an important recommended procedure that prevents buildup of explosive peroxides.

Septa (GC-Injection Port)

1. Septa with high thermal stability were used: Thermogreen (TM) LB-1 Septum, SUPELCO, INC.

2. The septa were stored in capped organic-free containers until use. They were inserted into the injection port using cleaned tools.

This procedure made it unnecessary to precondition the septa after installation in the GC or GCMS.

Field Sampling Procedures

Water Extraction using Sep Pak Cartridge

1. Luer-type syringe was fitted with a C18 Sep Pak (Waters Associates).

2. A clean glass jar was immersed to obtain a subsurface water sample.

3. This water was transferred into the barrel of the syringe. The plunger was inserted and the water was pushed through the C18 cartridge.

4. Step 3 was repeated until the back pressure buildup, due to filtered particulates plugging the cartridge, prevented further concentration

of organics on the C18 material. The total water volume was noted, usually 120-150 ml.

5. The cartridge was returned to its original foil packet. The foil was bent to give partial sealing protection. The collected samples were stored in a clean glass bottle that was sealed until analysis was ready to be performed.

6. Trials with varied amounts of isopropanol, methanol and tetrahydrofuran showed that 75-80 percent recovery was obtained following desorption with at least 1.5 ml of tetrahydrofuran; 2.5 ml was used in the actual procedure.

7. 1.7 ml of water was added to the extract from (6) to match the liquid composition to the initial carrier composition used in liquid chromatography.

Sediment--

1. Tenneco Pond and contiguous creek sediments. A small grab sampler was used to take surface samples. The sampler was opened and the sample was discharged into a scrubbed galvanized metal bucket. A clean metal scoop was used to transfer the moist sediment to clean glass jars.

2. Chester River. Sampling sites were selected near the main channel in order to obtain clay-rich specimens. A Van Veen grab sampler was used to bring up surface sediment samples. The sampler was fitted with a sliding panel to permit insertion of the coring tube. The clean brass coring tube was inserted to get a 10 cm vertical core of the uppermost sediment. The brass tube was sealed by placing a hand at the top. Then the cylindrical core was lifted out of the grab sampler. The core was released onto baked aluminum foil, and then stored in the foil in a clean glass container. The latter was stored for library purposes.

3. All sediment samples were transferred within 12 hours to storage at 4°C until ready for drying.

4. All sediment samples used in this study were subjected to the following drying procedure.

Sediment Drying by Isopiestic Dehydration

a. The collected sediment is homogenized in the sample container using a clean metal spatula.

b. A 10 g sample is spread evently onto the surface of a clean 12 cm watch glass using the same spatula.

c. An organic-free desiccator is charged with 0.25 kg Drierite in the desiccator at 23°C for 48 hours.

d. To complete the drying, the sediment is removed to repeat the drying process. The desiccant is re-dried by baking in the desiccator at 250°C for 12 hours. The partly dried sediment is then returned to the cooled freshly baked desiccant for another 48 hours.

e. The dry sediment is scraped into a clean glass mortar. It is then pulverized using a clean glass pestle, transferred to labeled vials, and stored at 4 °C.

Two solid substances will undergo a gas phase transfer of water until a final equilibrium vapor pressure is reached. The repeated exposure of moist sediment to repeated fresh charges of the desiccant Na_2SO_4 causes isopiestic dehydration. The advantages of this approach are as follows:

1. Water content in clay sediment is reduced from about 60 percent to about 1 to 2 percent on a dry weight basis.

2. Unlike the procedure of mixing the desiccant in with the sediment (Bulla, personal communication), the water is physically removed and there is less opportunity for organic contamination from the desiccant.

3. Unlike lyophilization, isopiestic dehydration is carried out at atmospheric pressure with 10^3-fold shorter gas phase collision distances and a corresponding less likelihood for sublimative loss of the measured analytes (DBP or DEHP).

Sediment Extraction

From Karrickhoff's work (1979), it is known that organic pollutants in sediment are associated with the organic carbon content, due principally to humic and other associated polymers. Based on this, the sorptive structure should be modeled effectively by n-octanol. Accordingly, a volative extraction solvent, similar to polarity to octanol, was chosen as more likely to give efficient extraction. Since water polarity is much higher than that of octanol, the partitioning model clearly infers the need to remove water before attempting the extraction of neutral organic compounds.

This approach differs considerably from the conventional Soxhlet extraction procedure applied to wet sediment. The latter also requires large solvent to sample ratios so an extra burden is implied in terms of needed solvent purity. We were advised by Dr. George Boughman (personal communication) that Soxhlet extraction tends to be incomplete due to slow diffusion of solvent through the sediment sample. For that reason, a thin coating of sediment is advised to line the extraction thimble. Unfortunately, this procedure boosts the solvent-to-sample ratio even further.

Sediment Extraction with Methylene Chloride

1. Two grams of sediment is weighed to the nearest mg and placed in 10 ml capacity vials with Teflon lined caps. The caps are screwed on tightly to prevent evaporation.
2. Five ml of methylene chloride, containing 0.40 ppm dimethoxyethyl phthalate (DMEP) as internal standard, is introduced using a Repipet apparatus. (A different amount of DMEP was used for the Tenneco Pong sample. This involved 4.0 ppm DMEP in CH_2Cl_2, but there was no subsequent evaporation.)
3. The vials are first agitated in a Vari-whirl mixer to suspend the particles.
4. The vials are placed in an ultrasonic bath. Circulating water is used to hold a temperature of approximately 30°C for 2 minutes.
5. The vials are centrifuged at 2,500 G for 15 min. This is done to remove suspended particles.
6. The supernatant liquid is drawn off and placed in a 5 ml capacity vial using a Pasteur pipet.
7. The extract is evaporated under a stream of purified nitrogen to a volume of approximately 0.5 ml. The nitrogen is purified by passing through a column of carefully extracted washed (Chester et al. 1976) XAD-2 resin.

8. Two hundred µl of isooctane is added to the vials to prevent evaporation to dryness. The extract is blown down to 0.20 µl. This gives an overall 25 x concentration (5 ml to 0.2 ml).

9. A procedural blank was prepared in triplicate for each experiment. Each contains the extracting solvent and internal standard but no sediment samples. Isooctane is added after the blanks have been sonicated and blown down. For LC analysis, tetrahydrofuran was substituted for the isooctane. These procedures are summarized in Tables 9 and 10.

TABLE 9. FIELD SAMPLE HANDLING

Procedure	Precleaning
Surface Grab Sampler	On-board High Pressure Water Hose
Obtain Core within Grab Sampler	Use brass tube precleaned by acid etch, soap, water. Swabbed with abrasive between samples.
Storage	Use baked glass bottles, aluminum cap liners. Hold for 14 days at 4°C.
Isopiestic Dehydration	Two 48-hour periods. Closed system
Sample Homogeneity	Sample grinding with baked glass mortar and pestle.
Storage	Dried sample stored at 4°C.

TABLE 10. METHODOLOGY

Step	Procedure	Reagents Added	Conditions
1	Isopiestic dehydration of sediment	Na_2SO_4 dried at 180°C	Closed system 22°C, 1 atm
2	Ultrasonic extraction with CH_2Cl_2	CH_2Cl_2 containing 0.4 or 4.0 ppm DMEP internal standard	Closed system t = 30°C
3	Centrifugation	None	Closed system t = 10°C 2500 x G
4	Evaporate solvent	XAD-2 filtered N_2: (a) Isooctane added for GC or GCMS (b) THF added, CH_2Cl_2 removed, for HPLC	Open system

Preliminary Liquid Chromatographic Analysis

High performance liquid chromatography has been reported for analysis of alkyl phthalate esters (Hellman 1978). This technique was adapted to confirm GC measurements on Tenneco Pond sediment, as a screen and to provide upper limit data on samples containing small amounts of alkyl phthalates. The Hellman method is based on adsorption chromatography. A more reliable method was developed based on partition chromatography using water-tetrahydrofuran as the carrier with bonded C18 as the stationary phase.

A surface sample of water from the environment was transferred onsite to the barrel of a baked glass Luer-type syringe. A Sep Pak C18 cartridge was already fixed in place. The water was then passed through the cartridge using hand pressure after inserting the plunger into the barrel of the syringe. This was continued until the back pressure became too great. Then the cartridge was returned to its original protective envelope, sealed, and labeled.

Preliminary laboratory tests had shown that THF (tetrahydrofuran) gave sharper peaks than either methanol or isopropanol which are widely used. The recovery of DEHP standard was about

74 percent by desorbing with 1 ml of THF, or 75-80 percent if > 1.5 ml were used. The final procedure called for 2.5 ml of THF. Then 1.7 ml of purified water was added so that the liquid sample composition matched the initial HPLC carrier make up. The liquid chromatograph 254 nm UV monitor (Waters Model 440) remained on scale using 25 µl samples of extract.

Similar tests were carried out using DTDP (ditridecylphthalate) and the results were quite similar except that a lower recovery, approximately 60 percent, was obtained.

The precision of the HPLC results were found to be linear with the amount of added standard to within 5 percent variability. This was observed during Sep Pak adsorption of 10 ppm DEHP dissolved in water samples ranging from 30 to 150 ml in volume.

Water analysis in Tenneco Pond and several sampling sites in the Chester River was attempted using C18-Sep Pak cartridges to concentrate the analyte. These cartridges were soon clogged by suspended particles so the sample volume was limited to 150 ml or less. GCMS showed that the liquid chromatographic (LC) technique alone in one instance gave an apparent but false identification of ditridecylphthalate. At trace concentrations, LC was considered valid for setting upper limits.

The final conditions for HPLC analysis were obtained using a linear carrier gradient proceeding from 60 percent THF/40 percent H_2O to 90 percent/10 percent over a 5.0 minute period. A flow rate of 2.0 ml/min was used.

Sediment extracts in dichloromethane were twice treated by adding THF and using purified nitrogen gas blowdown to remove the dichloromethane. Then makeup water was added, as before, to give the correct liquid solvent rates.

Quantitative Analysis of Sediment Extract using GC/MS-SIM

The Hewlett Packard 5992A GC/MS system was used for the analysis of sediment extracts. The microprocessor-controlled 5992A uses a jet separator to interface the gas chromatograph to a hyperbolic quadrupole mass filter. A 3-foot (90 cm) silanized glass column (packed with 3 percent SE-30 on Chromosorb W-AW-DMCS) was used. The column was temperature programmed from 140°C-250°C at 5°/min. The system was later converted to a SE-52 glass capillary column programmed from 160°C-275°C at 7.5°/min. Up to 6 ions can be monitoring during a chromatographic run in the selected ion monitoring mode. The base peak of DEHP, m/z 149 was monitored. Mass 59 was also monitored which is characteristic of the internal standard, dimethoxyethyl phthalate (DMEP). DMEP has a low mass 149 abundance which decreases its chromatographic interference with other possible phthalates. It is also likely to exhibit physical and chemical properties similar to other phthalates, and it is not produced commercially as a plasticizer. By adding DMEP to the extracting solvent, methylene chloride, it is susceptible to the same systematic errors during the analytical scheme as other phthalates being determined.

GC/MS Autotune Procedure

Each week "autotune" was run to adjust and to assess the condition of the GC/MS instrument. This procedure tunes the ion source and mass filter to produce a mass spectrum of perfluorotributyl amine (PFTBA) to meet certain minimum specifications. If these specifications, as recommended by the manufacturer, were not met, the problem was diagnosed and corrected before continuing the work.

GC/MS-SIM Calculation

The mass 149 chromatogram of all sediments taken from the Chester River show only two well-defined peaks at GC retention times that correspond to dibutylphthalate DBP, and dioctylphthalate. Quantitative analysis for each of these two compounds is based on monitoring mass 148 peak areas for DBP and DEHP, and mass 59 for the internal standard. Within experimental error, we verified the linearity typical in GC/MS-SIM analysis. Defining the following terms

C = concentration of solute in CH_2Cl_2 (mg/ml CH_2Cl_2)
S = area under mass-chromatographic peak (area units)
a = analyte (DEHP or DBP)
i = internal standard (DMEP)
k = response factor

we have

$$S_a = K_a \text{ and } S_i = K_i c_i .$$

The ratio k_a/k_i was found to be constant for total analyte injections less than 0.3 µg providing C_a/C_i were within a factor of 100 of unity. Then, it follows that

$$R = \frac{k_a}{k_i} = \text{constant} = \left(\frac{S_a C_i}{C_a S_i}\right) = \left(\frac{S_a C_i}{C_a S_i}\right).$$

The latter term was measured giving $k_a/k_i = 11.0 \pm 0.4$. It follows that

$$C_a = \frac{C_i}{S_i}\frac{S_a}{R} .$$

In order to calculate the weight fraction f_a of analyte in the sediment sample, the following relationship was used:

$$f_a\left(\frac{\mu g\ \text{of a}}{g\ \text{sediment}}\right) = C_a\left(\frac{\mu g\ \text{of a}}{ml\ CH_2Cl_2}\right)\ \frac{V_e(ml\ CH_2Cl_2)}{W_s(g\ \text{sediment})}$$

This uses the volume V_e of the final concentrate extract, and the initial weight W_s of dry sediment to provide the weight of DBP or DEHP. The units, µg/g, are the same as parts per million (ppm).

RESULTS AND DISCUSSION

Preliminary Results with HPLC

A detailed study (Gingras 1979) was made of the basis for applying modern liquid chromatography to analysis of field sediment and water samples for alkyl phthalates. Previously, Hellman (1978) had developed the use of adsorption liquid chromatography but this is prone to irreversible adsorption effects and lesser reproducibility. Amundson (1978) used reversed phase LC with Bondapak C-18 as the stationary phase with methanol plus 1 percent acetic acid as the carrier. More sharply defined peaks were obtained more rapidly with tetrahydrofuran-water mixtures, 60:40 linear programmed in a 5-minute period to 90:10 at 2.0 cm^3/ml flow rate.

Analysis was made by LC bases on preliminary test samples of phthalates as manufactured, named and provided to us by Tenneco. These are shown in Figures 8-12. The LC technique has notable stability but qualitative identifications can only be shown to be consistent or inconsistent with the LC retention volumes. The time of analysis was only 7 minutes, or less than half required by Anumdson. The resolution of a synthetic mixture of the three reference materials DOP, DIDP (diieodecylphthalte) and DTDP is illustrated in Figure 9. Later on in the study we established that Tenneco's "DOP" was actually DEHP.

Analysis of Tenneco Pond water was carried out using the Sep Pak technique and the results are shown in Figure 10. The presence of DOP 0.25 ± 0.15 ppm and DTDP (ditridecylphthalate, 1.5 ± 0.2 ppm) were estimated by LC alone. The presence of phthalate esters in downstream water samples could not be detected by LC so these will not be reported here. The first peak

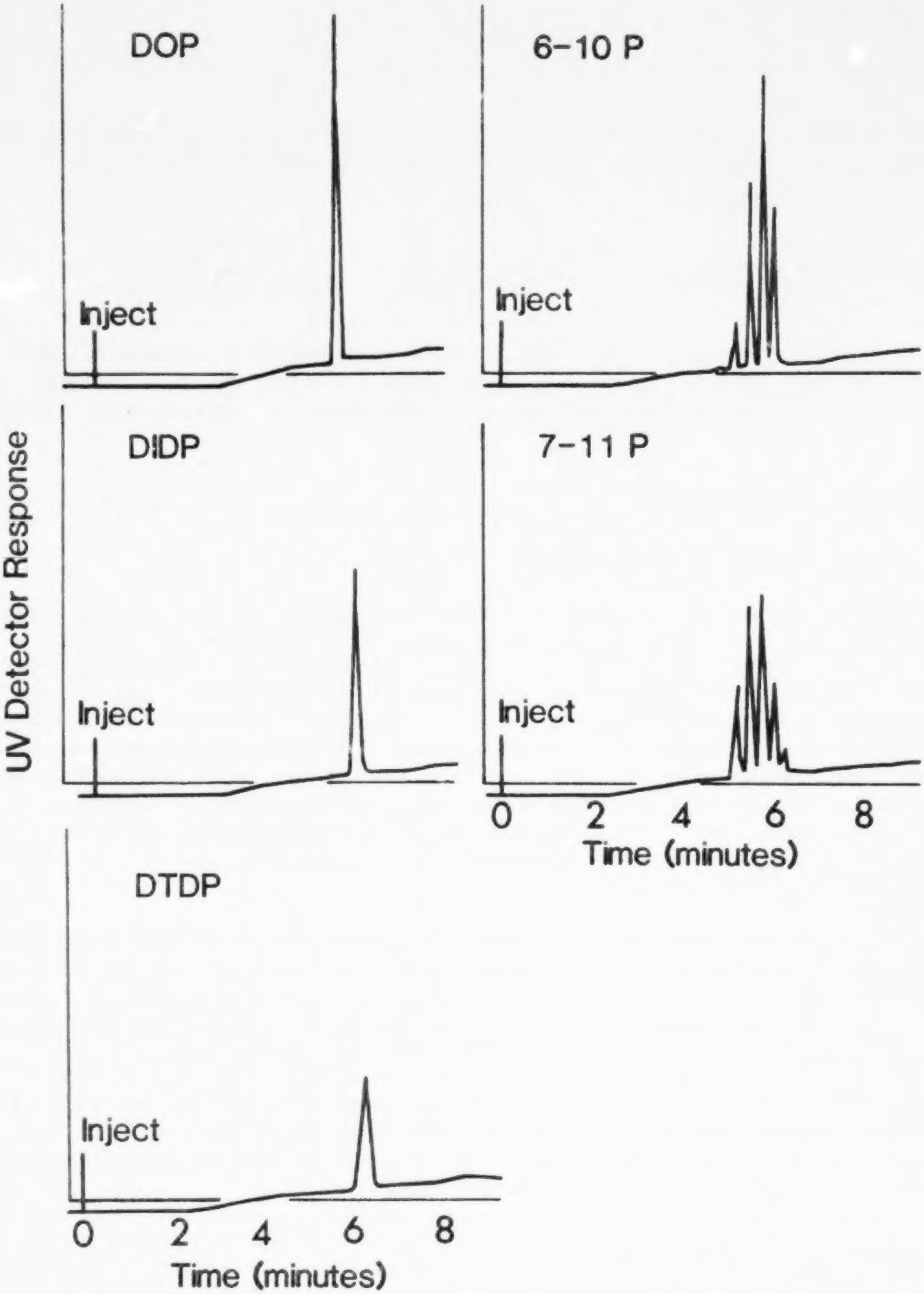

Figure 8. Liquid chromatography of Tenneco Products designated by Tenneco as Alkyl Phthalates, or mixtures (6-10P and 7-11P).

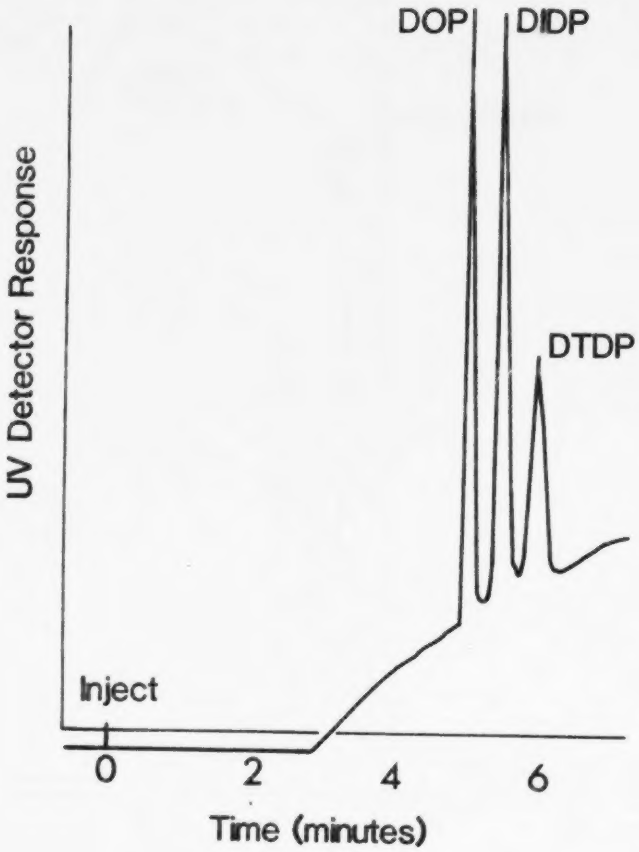

Figure 9. Liquid chromatogram of DOP, DIDP, and DTDP (synthetic mixture of Tenneco samples).

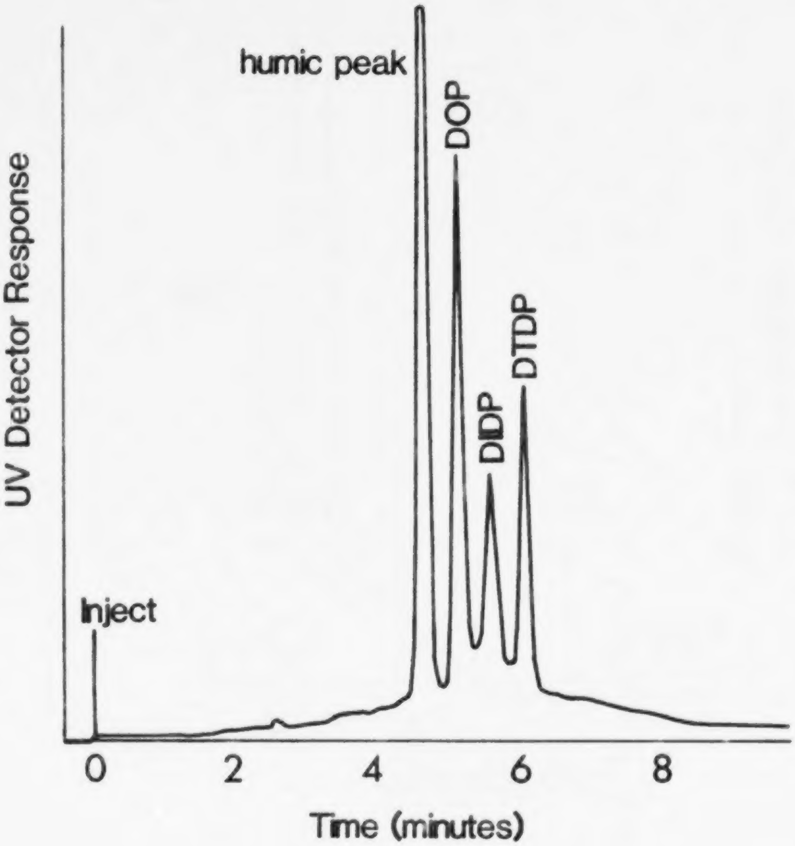

Figure 10. Liquid chromatogram of Tenneco pond water. Identification based on Tenneco reference samples.

in Figure 10 is likely to be due to the fulvic acid component which occurs in natural water and sediment extracts. Although a number of Morgan Creek and Chester River water samples were run by the same technique, the method was frustrated by early plugging of the Sep Paks. This prevented the obtaining of large enough samples to give the needed sensitivity.

Analysis was made by LC of Tenneco Pond sediment. The result is shown in Figure 11. The concentrations are so high that peak verification by GC/MS was readily demonstrated and quantitative interpretations are thus possible. The retention volumes are consistent with the presence of humic acid, of DEHP $(1.8 \pm 0.1) \times 10^3$ ppm and a second determination of $(1.3 \times \pm 0.2) \times 10^3$ ppm, of DIDP $(1.4 \pm 0.2) \times 10^3$ ppm and of DTDP $(1.9 \pm 0.2) \times 10^3$ ppm. The qualitative identifications by LC were each confirmed by GC/MS-SIM retention times of m/z 149.

A few miles downstream from Tenneco on Morgan Creek sediment samples were taken for LC analysis. Comparison was made to humic acid under the same conditions. These results are shown in Figures 12 and 13, respectively. It is clear that the natural background, the multiple peaks due to fulvic and humic acid constituents, seriously interfere with and prevent quantitative use of these LC results. Sediment samples taken from the Chestertown Bridge and from the Chester River (site 5, Figure 4) showed no evidence for DEHP, apparently less than 1.0 ppm. Artifact peaks were observed and these suggested exaggerated levels of DIDP and DTDP. The GC/MS technique gave qualitative but not quantitative confirmations of DEHP and DBP so these were concluded as present at both sites. The DBP peak position was found to be consistently obscured in the LC by the humic components. Significantly, this was the only alkyl phthalate which Tenneco did not make, but which is otherwise massively produced by U.S. industry. Second, certain peaks evident at the equivalent of <10 ppm levels by LC were found by GC/MS not to be phthalates. Attention was then directed to the GC/MS technique which was considered necessary for identification and measurement of DBP and DEHP at levels below 100 ppm. The LC technique offered preliminary qualitative utility and it was the more reliable for measuring DIDP and DTDP.

Preliminary Qualitative Results Using GC and GC/MS

A preliminary grab sample of Tenneco Pond sediment was obtained. The sediment was desiccated and then extracted by methanol using ultrasonic agitation. Centrifugation gave a clear filtrate which was then analyzed by gas chromatography. The result showed a single peak which accounted for >95 percent of all volatiles apparent by the FID detector.

Gas chromatographic retention times and mass fragmentation patterns showed clearly that the very conspicuous main organic volatile component in Tenneco Pond sediment is the same as the

DEHP reference sample which, in turn, matches the samples of DEHP from Chem Service, Inc. The mass fragmentation patterns showed closely similar abundance profiles at m/z 61, 70, 71, 83, 104, 112, 113, 149 (base), 150, 167, 168, and 279.

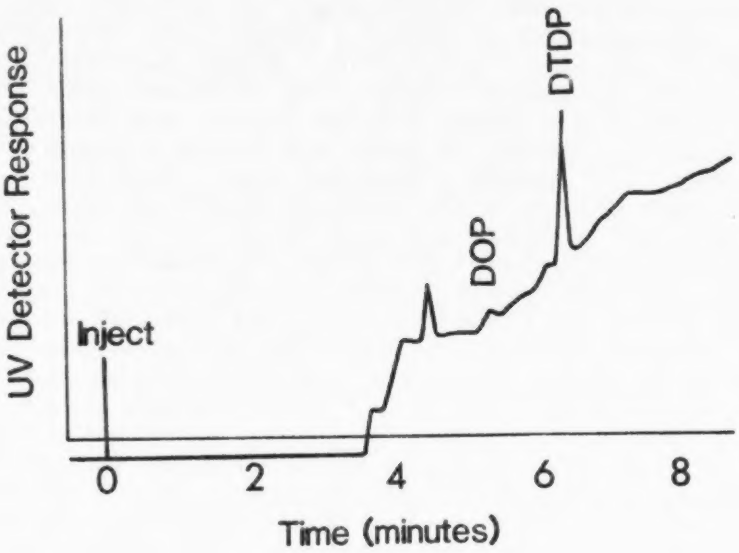

Figure 11. Liquid chromatogram of Tenneco pond sediment.

Reference data from the NIH/EPA library, recently published by the National Bureau of Standards, strengthened the assignment of DEHP.

DEHP was subsequently distinguished from DNOP by their g.c. retention times and mass spectra. There was no DNOP detected in the Chester River sediment. The use of glass capillary GCMS permitted a clear distinction (see p. 69).

Tests of the Methodology Based on GC/MS

A series of experiments were carried out in order to validate the method. Preliminary experiments showed that desorption of DEHP or DBP occurred more efficiently from dry rather than wet sediment. Very few wet extractions were carried out thereafter.

Rate of Extraction--

A series of extractions by dichloromethane of the dry working standard "R" (see Figure 7) were carried out. The DBP extraction was independent of the time given to the sonication step which was varied from 30 seconds to 4 hours. The results are shown in Table 11. The results show no apparent trend with time of sonication. Other experiments confirmed the finding

TABLE 11. EFFECT OF SONICATION ON DBP EXTRACTION BY CH_2Cl_2 FROM SAMPLE "R"

Sonication Time (min)	DBP Measurement (ppm)*
0.5	$0.78\overline{5}$
5.0	$0.72\overline{5}$, 0.66
30.0	0.75
120.0	0.79, 0.715
Average Value	0.74 ± 0.02 (± SDM, n = 6)
Standard Deviation	0.05
Standard Deviation of Mean	0.02
Internal Standard:	d-10-Anthracene

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* These preliminary results are to be used on the basis of their relative accuracy. Subsequent determinations of absolute DBP levels were found to be more accurate.

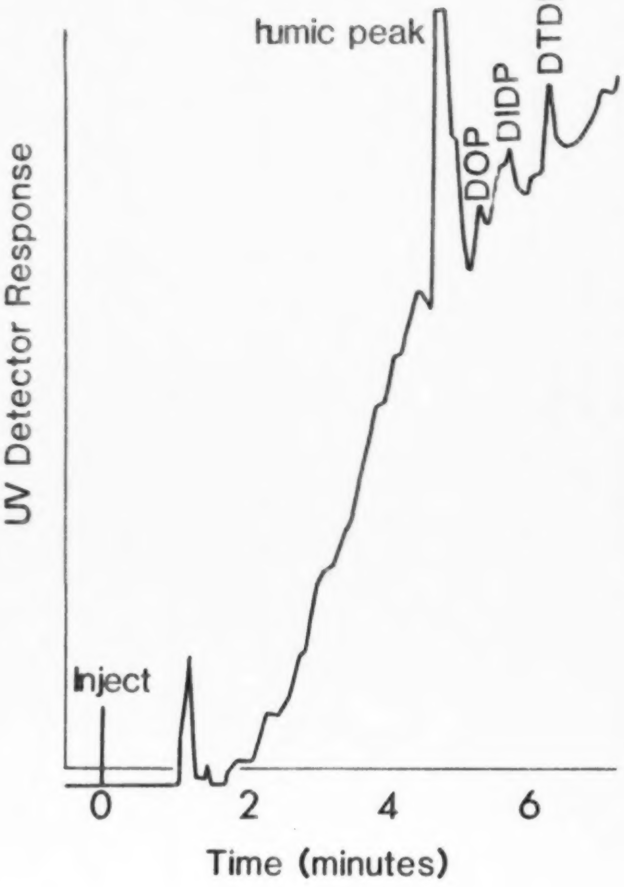


Figure 12. Liquid chromatogram of Morgan Creek sediment from Frye Farm a few miles downstream from Tenneco.

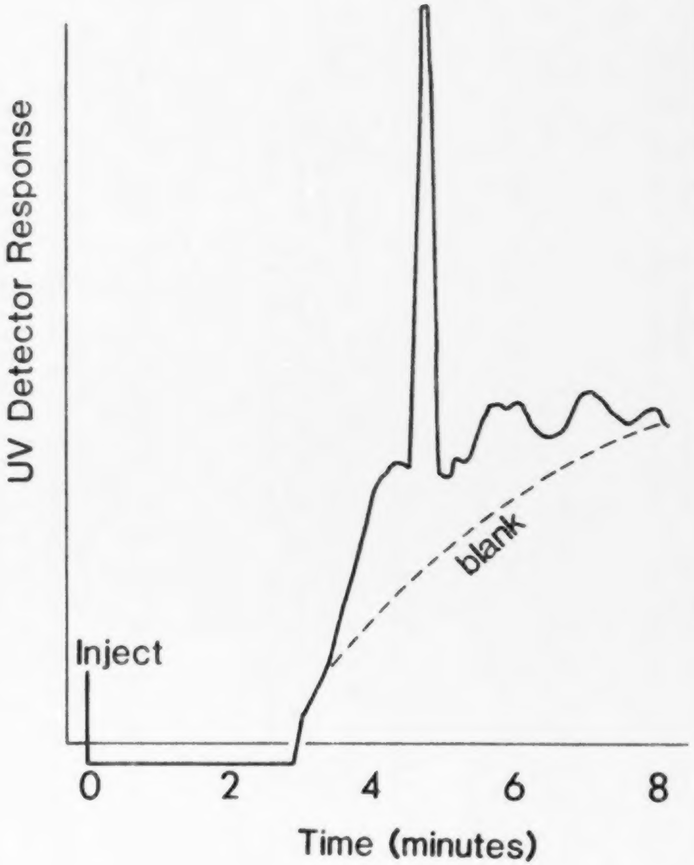


Figure 13. Comparison liquid chromatogram of humic acid.

that the desorption rate in CH_2Cl_2 was too rapid to be observed kinetically. The extraction time was fixed at 2.0 minutes. It should be noted here that reproducible time course studies were not demonstrated until we discovered the necessity of using cooling water (30°C) to prevent a temperature rise and subsequent loss problems during the extraction period.

Effect of Solvent--

Various solvents were tested for their extractive power. The conditions were identical to those used in Table 12. The results are shown in Table 12. It is clear that hexane gives decidedly poor extractive efficiency, while benzene, dichloromethane and methanol give quite similar extractive efficiency.

The choice of solvent depends upon more than the rate of extraction. The variation in total amounts of extracted organic matter was not measured. A simple color comparison of extracts, often clarification by centrifugation, showed that hexane exhibited least color, dichloromethane gave a visible light yellow color, and methanol showed a substantially deeper color. Analyses were performed on the extracts immediately after extraction. After a period of several weeks a gum would form in the dichloromethane extract and inconclusive tests suggested that dissolved organics might have been lowered in concentration as a result. Clearly, methanol would lend to a distinctly higher concentration of nonvolatile substances and these would be likely to foul the chromatograph. Hexane gave the cleanest extract but, of course, it has a poor yield. Benzene is toxic so this was ruled out. Therefore, dichloromethane was chosen for its high extractability, low toxicity, and ease of volatilization.

TABLE 12. COMPARISON OF ULTRASONIC EXTRACTION EFFICIENCIES OF VARIED SOLVENTS MEASURED ON WORKING STANDARD*

Solvent	Trials	Measured DBP (ppm)
Hexane	3	0.34 ± 0.07†
Benzene	3	0.65 ± 0.02
Dichloromethane	3	0.70 ± 0.01
Methanol	3	0.67 ± 0.03

* Based on a fixed ultrasonic extraction period of 2.0 minutes (Chester River Sample "R").

† Tolerance is expressed as the standard deviation.

Internal Standard: D-10 anthracene.

Recovery Tests--

Several experiments were carried out to show whether DEHP could be added to the measurement system and then recovered without serious loss. The results are summarized in Table 13, which is largely self-explanatory. The most serious loss seemed to occur when dichloromethane containing a DEHP spike was deliberately evaporated to remove all of the solvent. When this was done a 7 percent DEHP loss was observed. To prevent this in the actual procedure, a small amount of isooctane, a higher boiling solvent, was added to prevent the volatilization and no other measurements in the report involved total solvent volatilization exhibited here for test purposes.

TABLE 13. DEHP RECOVERY MEASUREMENTS USING DICHLOROMETHANE

Sample	Procedure	Trials	Percent Recovery
Spiked Solvent*	Remove 95% of solvent. Add fresh solvent to bring to initial volume.	3	100 ± 2
Spiked Solvent*	Remove 100% of solvent. Add fresh solvent to bring to initial volume.	3	93 ± 1
Spiked Blank† (attapulgite)	Remove 98% of water by desiccation.	4	98 ± 1
Spiked Blank (attapulgite)	Ultrasonic extration by dichloromethane		

* 5 ml of CH_2Cl_2 containing 1 mg of added DEHP.

† 1 g of Attapulgite containing 0.2 mg of added DEHP.

N.B.(1) Internal standard = d-10-anthracene
N.B. Reagent blanks are as follows:

CH_2Cl_2 = 20 ± 10 ppb of DEHP

Attapulgite = 10 ppb of DEHP

Effect of Varied Methodology and Combinations--

The establishment of a 2.0 minute sonication procedure for extracting dry sediment with dichloromethane was subjected to a series of tests to measure the completeness of the extractive process. The same working standard "R" was used. The sonication procedure is the same as that previously described. The

Soxhlet extraction procedures are as follows:

Wet Soxhlet Procedure

1. The extraction thimble 43 x 123 mm was extracted with methylene chloride for 24 hours before use.
2. A clean 2000 ml flask was charged with 500 ml of methanol containing approximately 5 µg of the internal standard, d-10 anthracene ($C_{14}D_{10}$).
3. The thimble was loaded with approximately 90 g of wet sediment (60 percent moisture content). The sediment was smeared onto the walls of the thimble cylinder to provide more intimate contact with the solvent.
4. After 24 hours the methanol was emptied into a storage container and replaced by 500 ml methylene chloride and an additional 5 µg of d-10 anthracene.
5. The extraction was continued for at least 48 hours, when the extract solution in the thimble chamber became completely clear.
6. The solvents were distilled off using Buchi Rotovapor R. until approximately 30 ml of aqueous extract remained.
7. This aqueous liquid was extracted with 5 volumes of methylene chloride. This extract was concentrated to 5 ml using the Rotovap. Further evaporation was done using a stream of nitrogen (XAD-2 filtered).
8. A blank was prepared by duplicating the procedure described above, but without the sediment samples.

Dry Soxhlet Extraction--This procedure is the same as the wet Soxhlet extraction procedure except for the following modifications.

1. The sediment was first dried by desiccation to a moisture content of 1-2 percent.
2. Approximately 40 g of dried sediment was added to the previously extracted thimble.
3. Only methylene chloride was used as extracting solvent and the extraction duration was 72 hours.

4. The methylene chloride extract was concentrated directly to 5 ml using the Rotovap followed by a stream of nitrogen. No solvent extraction step was needed.

The results of the various extraction tests are as follows:

Test 1

A comparison of the efficiency of ultrasonication and Soxhlet extraction is shown in Table 14. Ultrasonication extracted greater amounts of phthalates than Soxhlet extraction from the same sediment collected at the mouth of the Chester River. The incompleteness of the Soxhlet method is believed to be the result of the lower eddy diffusion of solvent in the compacted solid sediment. With particular regard to the time course study, it is felt that the present experiments give a sound basis to reject the Soxhlet technique on the basis of its greater proneness to contamination, its incompleteness and the high cost associated with slow rate and need for laborious repetition.

Test 2

A second and more stringent test of the sonication technique was carried out. Sonicated material was rinsed free of retained extracting liquid, and the samples were then subjected to re-extraction. The results are shown in Table 15.

The repeated use in test 26 of sonication drew a blank--no evidence for further extraction was observed. Essentially the same finding was observed when the sonicated sample was washed free and then subjected to Soxhlet extraction.

Test 3

A comparison was made of wet Soxhlet and dry sonicative extraction of Tenneco Pond sediment. In general, these more heavily polluted samples of sediment seemed to be more easily extracted so that is not a stringent test.

Analysis of the Internal Standards--The use of dimethoxyethyl phthalate (DMEP) seemed ideal in the sense that its gas chromatographic elution time fit into a window that caused no interference with the DBP or DEHP measurements. Further, its chemistry was parallel to that of the other phthalate esters. However, we eventually became aware that our procedural blank levels were significant: 0.10 ± 0.04 (n=6) ppm for DBP and 0.28 ± 0.22 (n=3) ppm for DEHP. Direct analysis of the DMEP standard revealed the cause since it contained 4 percent DBP and 11 percent DEHP! These are not serious interferences since the

TABLE 14. COMPARISON OF METHODS FOR EXTRACTION OF PHTHALATE ESTERS FROM CHESTER RIVER, MOUTH SEDIMENT. TEST 1.

Extraction Method	Amount Extracted, ppm, and Standard Deviation		
	/ DEP	/ DBP	/ DEHP
Ultrasonication	0.19 ± 0.03	0.36 ± 0.07	0.40 ± 0.06
Soxhlet, dry	0.10 ± 0.03	0.33 ± 0.14	0.34 ± 0.06
Soxhlet, wet	0.05 ± 0.10	0.26 ± 0.07	0.21 ± 0.12

Re-extraction by ultrasonication of sediment from all three methods yielded less than 1 percent of the first extraction value for all three phthalates.

TABLE 15. TEST OF VARIED EXTRACTION AND RE-EXTRACTION PROCEDURES

Sample	Test	Method	Sediment State	Trials	Result (ppm)
Chester River Sample "R"	1	Soxhlet and sonication	Dry and wet	3	See Table
(same)	2a	Soxhelt	Dry	3	0.9±0.1(DBP)
		Re-Sonication	"	3	0.04±0.02(DBP)
(same)	2b	Soxhlet	"	4	0.7±0.1(DBP)
		Re-Sonication	"	3	0.02 (DBP)†
		Re-Soxhlet	"	1	0.02 (DBP)†
Tenneco Pond	3	Soxhlet	Wet	1	1.0x10^3(DEHP)
		Sonication	Dry	5	(1.2±0.1)x10^3 (DEHP)

± Uncertainties are expressed as standard deviations of the mean values (rounded up).

† i.e., none detected.

Internal Standard: D-10-anthracene

calculations used a conventional blank subtraction step. However, the use of the contaminated DMEP was not considered to be desirable and another standard was introduced.

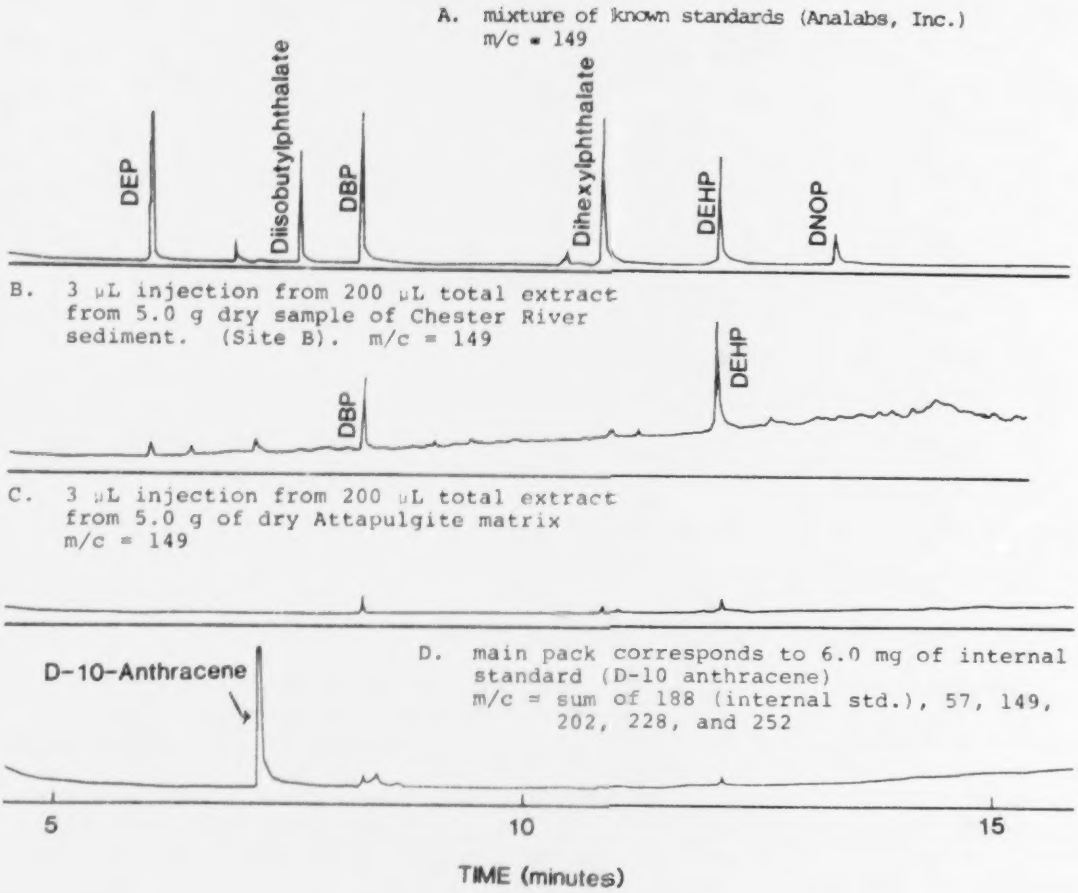
Since the method verification and environmental measurements were made at different times, we have been careful to note the internal standard for each experimental series.

The more recent work involved the use of an excellent internal standard, D-10-anthracene, $C_{14}D_{10}$. A small amount of DBP and a negligible amount of DEHP were observed with the anthracene standard. A correction for the DBP in the average blank was used in the caluculations. The use of $C_{14}D_{10}$ standard allowed more reliable measurements in the sub-ppm region. The basis for these statements, for the GCMS capability for discriminating between DEHP and DNOP, for the virtual absence of DNOP from sediment, and for the purity of the D-10-anthracene internal standard, i.e. illustrated in Figure 14.

Interlaboratory Comparisons of Split Sediment Samples

Two samples were carefully homogenized and sent to Dr. William Budde (EPA - Cincinnati) for interlaboratory comparison. The samples are the Chester River working standard "R" and a Tenneco Pond sample. The results are shown in Table **16.** A much closer agreement exists between the Tenneco Pond than the poor results obtained on the initial "R" sample. Since this involved two essentially independent experiments on the two unrelated samples and standards, it was decided to accept the results as validative for Tenneco Pond and to reject the results on sample "R" as nonvalidative.

The interlaboratory comparison was repeated using a fresh sample from our working standard "R." Samples were sent with and without use of our drying procedure. In addition, a spiked blank sample containing 200 ppm DEHP was sent for comparison measurements. Measurements on the split portions that were saved for that purpose were repeated. The results are given in Table 16. The results show reasonable agreement between the two laboratories. It should be noted that the results from EPA-Cincinnati seem to be higher than our results, and this remaining problem is still under study. However, the main purpose of demonstrating comparability has been achieved. A chronological account of interactions with Dr. William Budde (EPA-Cincinnati) is presented in Appendix B.



A. mixture of known standards (Analabs, Inc.) m/c = 149

B. 3 μL injection from 200 μL total extract from 5.0 g dry sample of Chester River sediment. (Site B). m/c = 149

C. 3 μL injection from 200 μL total extract from 5.0 g of dry Attapulgite matrix m/c = 149

D. main pack corresponds to 6.0 mg of internal standard (D-10 anthracene) m/c = sum of 188 (internal std.), 57, 149, 202, 228, and 252

Figure 14. Comparison of GCMS-SIM chromatograms.

TABLE 16. INTERLABORATORY COMPARISON OF DEHP MEASUREMENTS OF SPLIT SAMPLES*

Samples	Method	This Laboratory DEHP (ppm) see note d	EPA-Cincinnati DEHP (ppm) (n)	
Chester River, "R"	GCMS	0.097±0.03 (5)	58	see note a.
			0.3	see note b.
			0.4	see note c.
Spiked Attapulgite (dry)	GC GCMS	200±10 (5)	121	
Tenneco Pond West (dry)	GC	1200±100 (5)	1700	

Notes:

a. Initial determination. This errant result necessitated the subsequent repeating the entire analysis using freshly split samples.

b. Dried by this Laboratory.

c. Dried by EPA-Cincinnati.

d. Reagent blanks measured DEP 0.0005 ppm, DBP 0.001 ppm, DEHP 0.003 ppm.

* Further details are given in Appendix B.

Tenneco Pond and Chester River Sediments

Sediment analyses for DBP and DEHP were measured using the GC (Tenneco Pond) and GCMS (Chester River) techniques. The results are presented in Table 17. The samples in all cases were surficial--top 10 cm--that were thoroughly homogenized prior to drying and subsampling. The results are persented along with percentage of organic carbon [HCl (0.1M) treatment was used to remove carbonate] and percentage of water. All results are presented on a dry-weight basis.

Two samples were taken from Tenneco Pond which is roughly oval in shape. These separate samples were obtained near the center axis of the pond about one third of the distance from either end: these are labeled East and West.

It is clear that the pond sediment is quite substantially polluted with 0.15 percent DEHP. The previously cited LC results which seem to be much better for the larger alkyl phthalates show similar amounts of other compounds: 0.14 percent DIDP and 0.19 percent DTDP. The sum is nearly 0.5 percent on a dry-weight basis.

The total content of organic phthalate esters in Tenneco Pond can only be guessed at since the vertical concentration distribution was not determined in this work. For a depth of 0.04 m, the Tenneco Pond sediment volume for 40 x 10^3 m^2 of surface area is 1.6 x 10^3 m^3, or roughly 6.4 x 10^5 kg on a weight basis. The measurements are thus compatible with the presence of 960 kg of DEHP, 900 kg of DIDP, and 1200 kg of DTDP, or a total of about 3,000 kg of these three organics.

As discussed earlier, the State discharge permit allows Tenneco to release total organic extractables at a rate of 2000 kg/year. It would appear that Tenneco discharge may be operating rather near the permit, depending on where the measurement is made: plant discharge or outfall from the dam. The pond is clearly acting as a secondary waste treatment facility, and it is obvious that less organics have been flowing out of the pond than those that enter. The DBP content is low because Tenneco has rarely manufactured it.

The fact that the pond levels are now high should be interpreted as a warning. As the pond sediment becomes increasingly saturated with these organics, they may eventually move out into the Morgan Creek conduit. Since the linear flow rates are much higher and the creek bed is rather narrow, it is important to consider the eventual saturation of the present sorptive capacity of the pond sediment. At a future time the allowed Tenneco discharge, 2000 kg/year, may become more likely to make a direct transit from the factory site to the Chester River, and to possibly high localized concentrations in the river sediments. If this situation is allowed to continue without any further restraint, one can not help but visualize a more pessimistic future for the sediment beds in the Chester River.

The Chester River sediments exhibit ranges of 0.020 to 0.064 ppm for DEHP and 0.23 to 0.85 ppm for DBP. Stations 3, 4, 5 and 6 were deliberately localized at the apex of the oyster mortality. The data are not marked by major apparent differences from the site "R" at the river mouth. Since healthy oyster beds have been maintained downstream of sited 4-6, there is no framework provided by the present data to assign alkyl phthalates as causally related to the 1973-75 oyster mortality. Of course, this is purely circumstantial reasoning, and the results can not be used to rule out the possibility. However, no oyster samples were saved.

TABLE 17. DETERMINATION OF SEDIMENT COMPOSITION

Site	Lat/Long	%H_2O	% C	Method	Alkyl Phthalate Concentrations*			DEHP/DEP
					DEP (n) ppm†	DBP (n) ppm†	DEHP (n) ppm†	
Tenneco Pond East	39°12'15" 76°04'45"	52	4.0	GC LC LC			1.5x10^3(4) (1.3±0.2)x10^3(4) (1.8±0.3)x10^3(4)	
Tenneco West	"	52	2.6	GC		0.2±0.1(5)		
Tenneco Pond average value								8000±4000
Frye Farm (Morgan Creek) at Rt. 314		1	1.6	GCMS	0.027(2)	0.013±0.007(4)	3.9±1.4(3)	
Morgan Creek at Rt. 291			3.8	GCMS	0.014(2)	0.076±0.057(6)	0.050±0.014(6)	0.7±0.4
Chester-town Bridge			1.3	GCMS	0.016±0.001(3)	0.049±0.012(6)	0.020±0.009(5)	0.4±0.2
Site 7	39°09'13" 76°04'13"	52	2.8	GCMS	0.073±0.021(4)	0.23±0.14(4)	0.064±	
Site 6	39°05'44" 76°09'15"	57	2.8	GCMS	0.040(2)	0.60(2)	0.053(1)	

(continued)

TABLE 17. (continued)

Site	Lat/Long	%H_2O	% C	Method	Alkyl Phthalate Concentrations*			DEHP/DEP
					DEP (n) ppm†	DBP (n) ppm†	DEHP (n) ppm†	
Site 5	39°05'52" 76°08'49"	51	2.3	GCMS	0.042(2)	0.51±0.13(3)	0.039(1)	
Site 4	39°05'15" 76°11'03"	53	2.4	GCMS	0.075(1)	0.85(2)	0.050(1)	
Site 3	39°04'12" 76°09'55"	49	2.4	GCMS	0.025±0.002(3)	0.46±0.23(3)	0.044(2)	
Average value in mortality zone (Site 3-7)					0.05±0.02(12)	0.5±0.3(14)	0.05±0.02(9)	0.1±0.07
Site 2	39°02'18" 76°09'55"	58	3.4	GCMS	0.065±0.001(3)	0.57±0.09(3)	0.071(1)	
Site 1	38°59'31" 76°12'50"	57	2.9	GCMS	0.029(2)	0.47(1)	0.11±0.02(3)	
Site R Chester River Mouth	39°2'54"	65	2.9	GCMS	0.014(2)	0.033±0.017(5)	0.097±0.031(5)	3±1

† error values are only given for n>2

± DnOP (Di-n-octyl phathalate) was not found in Chester River sediment (<0.001 ppm)

Reagent blanks measured: DEP<0.0005 ppm, DBP<0.001 ppm, DEHP<0.0003 ppm

* Other alkyl phthalates found in Tenneco Pond (East) DIDP(1.4±0.2x10^3(4); DTDP(1.9±0.2)x10^3(4)

ANALYSIS OF PHTHALATE ESTERS IN OYSTER TISSUE

Procedure

1. Whole oysters were collected from the Chester River and pooled according to sampling site. The number ranged from 4 to 8 individual oysters per site.

2. The pooled tissue was homogenized with a Virtis "23" homogenizer for one minute at low speed followed by two minutes at medium speed.

3. The homogenized tissue was then disrupted with a Branson W185 ultrasonic probe for 10 minutes. Light microscopy showed that this caused complete cell disruption.

4. The ultrasonic probe was cleaned by running the device twice in distilled water and once in methylene chloride. The homogenizer flask and blades were then rinsed with water, methanol, and methylene chloride. Blanks of water were run between tissue preparation to check for contamination.

5. Twelve to fifteen g of tissue from each site plus two controls prepared from commercial oysters were placed in 50-ml Erlenmeyer flasks, shell frozen, and placed in a vacuum desiccator.

6. The desiccator was attached to a Virtis lyophylizer and the dry tissue was dried in 24 hours.

7. The dried tissue was removed from each flask, pulverized with a mortar and pestle, and stored at 3°C.

8. Two hundred mg of dried tissue was placed in 10-ml vials. A 10 μg aliquot of anthracene d-10 was added with a Corning disposable micro pipette (± 0.5 percent accuracy). Five ml of methylene chloride was added.

9. The tissue was extracted using ultrasonic agitation (Bransonic 220 water bath) for 5 min.

10. The vials were centrifuged at 4,000 rpm for 15 min. to sediment the remaining tissue residue. The supernatant was drawn off. The sample was concentrated by solvent evaporation to a volume of approximately one milliliter.

11. The concentrated extract was injected directly in the GC-MS with a 25M capillary column coated with SE-52.

Samples

Oyster samples from the Chester River were collected July 2, 1979 by personnel of the Chesapeake Biological Laboratory. The samples were frozen for subsequent delivery. They arrived in jars which had cracked presumably during the freezing process. The extent of possible contamination, however, was considered minimal.

Oyster sample CBL-1 was collected at Buoy Rock corresponding to sediment site 1. Sample CBL-2 was from Spaniard Point where sediment sample 6 was collected. There is no equivalent sediment site for the CBL-3 sample collected at Ferry Bar. A fourth sample from Love Point contained too few oysters to analyze. A homogenate of seven commercial oysters from northern Chesapeake Bay was used for reference purposes. Unspiked samples as well as samples spiked with DEP, DBP and DEHP were prepared from this.

Results

Oysters contain approximately 80 percent water and 2 percent lipid. Both of these tend to interfere with the analytical determination. The water must first be removed before considering extraction of the phthalates with methylene chloride. The lipid content is readily soluble in methylene chloride, as are the alkyl phthalates. An attempt was made to dry the oyster tissue using the same method successfully used for wet sediment. The tissue homogenate was spread as thin layer on a watch glass and placed in a desiccator containing sodium sulfate. This method was discarded because a bacterial bloom tended to form on samples prepared in this way. However, the controls from commercial oysters were free from this mold-like growth. It is suspected that these oysters are thoroughly rinsed and an anti-bacterial preservative is added during processing.

A more rapid drying method was needed. The preferred alternative was lyophilization or "freeze-drying." Spiked and unspiked controls were first freeze-dried to evaluate the method. The spiked samples contained 20 ppm each of DEP, DBP and DEHP. The extracts of these samples were injected directly into a glass capillary gas chromatograph with an FID detection. Numerous, partially resolved peaks resulted but the spiked peaks were not evident. GC-MS was used to identify the four major sets of peaks. The first large peak was identified by matching its mass spectrum to reference spectra as palmitoleic acid and closely related compounds that elute at 200°-203°C. The second large set of peaks eluting at 215°-217°C was identified oleic acid. The third (228°-232°C) and fourth (244°-248°C) groups are linoleic acid and linolenic acid, respectively.

The extract were analyzed for phthalate ester using the selected ion monitoring mode. Chromatograms of the spiked and unspiked oysters are shown in Figures 14 and 15. Even though percent abundance of m/e 149 is linolenic acid is 3 percent, its high concentration in the injected extract makes it clearly evident in the m/e 149 selected ion monitoring team. Indeed, all four sets of peaks similarly appear in the m/e 149 mass chromatogram. While the DEP and DBP are resolved from interfering peaks, DEHP co-elutes with the linolenic acid group. The DEHP concentration must be greater than 2 ppm in order to make the interfering background insignificant and obtain meaningful quantitative results.

The recoveries of DEP, DBP and DEHP from spiked (20 ppm) oyster tissue takers through the entire drying and extraction procedure were 60 ± 6, 96 ± 5, and 100 ± 15 percent, respectively. The smaller recovery for DEP is assumed to be due to volitization during the freeze drying process.

The measured levels of alkyl phthalates in oyster tissue are reported in Table 11 along with values for sediment samples taken from the same zone.

As discussed earlier, the octanol-water partitioning model predicts that the hydrophobic phthalates will be concentrated in the total lipid portions of the oysters relative to the surrounding water. We did not measure lipid content per se, but the oyster and sediment values were compared on the basis of the measured total organic carbon content which was measured. The oyster contained a 16-fold higher concentration of organic carbon than the sediment. Accordingly, the alkyl phthalate concentration is predicted by the partition model to be approximately 16-fold higher than that in the sediment. This prediction does not consider any mechanism other than simple partitioning. Table 17 shows that the oyster-to-sediment ratio of total phthalate ester residue is consistent with this model. At the two sites where contiguous oyster and sediment samples were obtained, the ratio is 16:1 (Buoy Rock) and 23:1 (Spaniard Point). This suggests that the surficial analysis of stratified (anoxic, nonbioturbated) taken nearby oyster beds may provide a basis for predicting the concentration of phthalates in the nearby oysters. It is also apparent that the level of phthalates in the oysters from the Chester River do not significantly differ from the reference sample of commercial oysters, also taken from the northern Chesapeake Bay.

Table 18 shows that the measured levels of alkyl phthalates in oysters range from 0.45 to 1.5 ppm (wet basis). The U.S. Bureau of Sport Fisheries and Wildlife reported recently (Mayer et al. 1972) a range of 0.2 to 3.2 ppm of alkyl phthalates in channel catfish and walleyes from various parts of North America. Also, a survey of 145 catfish farms (Haudet 1970)

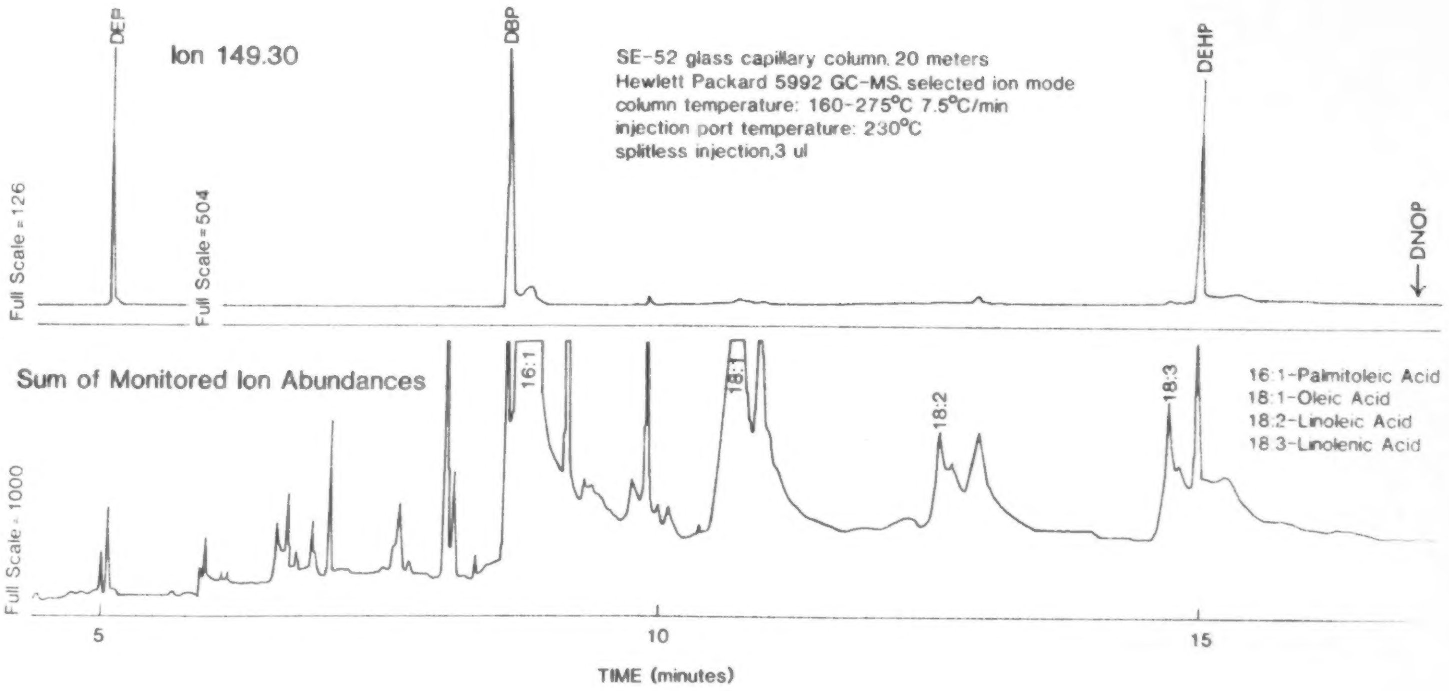
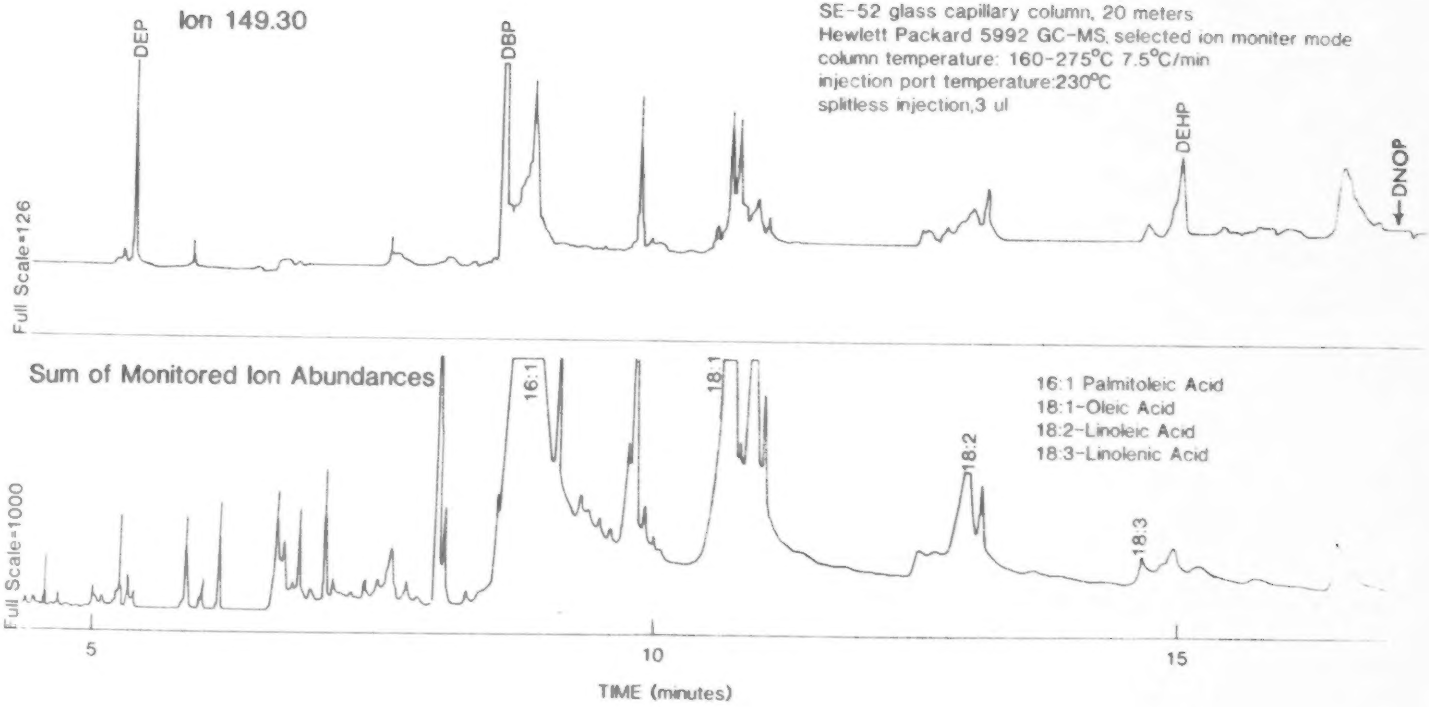


Figure 15. Oyster tissue extract (spiked with 20 ppm DEP, DBP, and DEHP).



SE-52 glass capillary column, 20 meters
Hewlett Packard 5992 GC-MS, selected ion moniter mode
column temperature: 160-275°C 7.5°C/min
injection port temperature:230°C
splitless injection,3 ul

Figure 16. Oyster tissue extract.

TABLE 18. CONCENTRATION OF PHTHALATES IN OYSTERS AND RELATED SEDIMENTS*

Site	Lat/Long	Sample	%H_2O	% C	DEP, ppm (n)		DBP, ppm (n)	
					dry†	wet‡	dry†	wet‡
CBL-1 Buoy Rock	38°59'31" 76°12'54"	Oyster	82	44	4.1±0.3(3)	0.74±0.05(3)	3±1(3)	0.5±0.2(3)
(sediment site 1)	38°59'13" 76°12'50"	Sediment	57	2.9	0.029(2)	0.012(2)	0.47(1)	0.20(1)
CBL-2 Spaniard Point	39°05'53" 76°09'15"	Oyster	82	44	3±3(3)	0.5±0.5(3)	5±2(3)	0.9±0.4(3)
(sediment site 6	39°05'44" 76°09'15"	Sediment	51	2.8	0.040(2)	0.017(2)	0.60(2)	0.026(2)
CBL-3 Ferry Bar no equivalent sediment site	39°00'08" 76°14'52"	Oyster	82	44	<0.05(3)	<0.01(3)	<0.05(3)	<0.01(3)
Control Commercial Oyster N. Chesapeake Bay		Oyster	82	44	2±2(3)	0.4±0.4(3)	7±4(3)	1.3±0.7(3)

(continued)

TABLE 18. (continued)

Site	Lat/Long	Sample	DEHP, ppm (n) dry†	DEHP, ppm (n) wet‡	Total C_T dry	$\frac{C_T \text{ oyster}}{C_T \text{ sediment}}$	$\frac{\% \text{ C oyster}}{\% \text{ C sediment}}$
CBL-1 Buoy Rock	38°59'31" 76°12'54"	Oyster	3±6(3)	0.5±1.5(3)	10±6(9)	16±9	16
(sediment site 1)	38°59'13" 76°12'50"	Sediment	0.11±0.02(3)	0.047±0.009(3)			
CBL-2 Spaniard Point	39°05'53"	Oyster	8±5(3)	1.4±0.9(3)	16±9(9)	23±13	16
(sediment site 6)	39°05'44" 76°09'15"	Sediment	0.053(1)	0.023(1)	0.7		
CBL-3 Ferry Bar no equivalent sediment site	39°00'08" 76°14'52"	Oyster	<2(3)	<0.4(3)	<3(9)		
Control Commercial Oyster N. Chesapeake Bay		Oyster	<2(3)	<0.4(3)	<11(9)		

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* Refer to page 76 for discussion of alkyl phthalate toxicity to aquatic organisms. Based on use of D-10 anthracene as the internal standard.

† Based on whole, dry oyster tissue.

‡ Based on whole, wet oyster tissue.

Reagent blanks measured: DEP<0.0005 ppm, DBP<0.001 ppm, DEHP<0.0003 ppm.

revealed that 95 percent of the fish analyzed contained DEHP residues with an average DEHP concentration of 3.15 ppm.

The alkyl phthalate measurements reported here can be compared, at least indirectly, to available toxicity data. Chemical toxicity results for aquatic organisms are usually presented as LC_{50} values (LC_{50} is the estimated concentration where the toxic substance in the surrounding water will kill half the population of exposed organisms within a certain time period, usually 96 hours.) Since the present study presents values for sediment concentrations, not water, an estimate of the water concentration using the octanol/water partitioning model was made. The partitioning constant (Kow) of phthalate esters is estimated to be $10^3 - 10^4$ (TSCA 1978). The average sediment values on an organic carbon basis (C_{OC}) for the mortality zone are 2 ppm, 20 ppm and 2 ppm (μg/g organic acid) for DEP, DBP, and DEHP, respectively. The estimated ester concentrations in water are, therefore, 0.2 ppb, 2 ppb, and 0.2 ppb (μg/L), respectively, for DEP, DBP and DEHP. Mayer and Sanders (1973) reported that acute 96 hours LC_{50} values for DBP with fathead minnor, channel catfish, rainbow trout, scud and crayfish fell between 730 and 10,000 ppb (μg/L). The LC_{50} concentrations for DEHP were estimated to be above 10,000 ppb. The estimated water concentrations in the Chester River are currently significantly less than these values. In addition, the average alkyl phthalate concentrations in oyster tissue in the Chester River range from 3 to 8 ppm. This is 1000-fold lower than the acute toxicity of alkyl phthalates for rats (intraperitonel) which ranges from 3-14 g/kg (percent). In comparison, the LD_{50}'s for organochlorine pesticides in rats are three orders of magnitude lower (20 - 300 mg/kg) than alkyl phthalates (TSCA 1978).

Chronic toxic effects of alkyl phthalates on aquatic organisms appear at much lower levels than acute toxicity. Mayer and Sanders (1973) reported that a concentration of only 3 ppb of DEHP in the water was sufficient to significantly decrease growth and reproduction of the crustacean *Daphnia magna*. Zebra fish and guppy reproduction was also impaired when their food was spiked with 50 and 100 ppm of DEHP. Various effects of alkyl phthalate have been reported for brine shrimp, goldfish and ring doves at concentrations varying from 3 to 10,000 ppb.

No chronic or acute toxicological data are available on the effects of phthalates on oysters. Martin and Roosenburg (1979) studied oyster mortality at 10 stations in the Chester River. No significant mortality occurred during the four-month period of observation. However, during July and August five stations furthest upstream had dieoff of the fouling organisms and reduced growth rates of the oysters. Ninety-six hour acute toxicity studies were performed on golden shiners and crayfish in streams receiving effluents from the Campbell's Soup plant, the Tenneco, Inc. plant and the Chestertown sewage treatment plant. No significant oyster mortality was observed.

It appears that a comparison of our measurements with the known toxic effects of phthalate esters on oysters and other aquatic organisms is unable to explain the Chester River oyster mortality during 1973-1975. However, there is enough evidence to cause concern over long-term effects on aquatic organisms especially with expected future increases production of alkyl phthalates.

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SECTION 6

MICROBIAL TRANSFORMATION OF TIN

EXPERIMENTAL PROCEDURES

Sampling

Samples were taken in the Chester River at Buoy Rock (latitude 38°59'33"N, longitude 76°12'27"W), which is a productive bar; at Spaniard Bar (latitude 30°05'57"N, longitude 76°08'55"W), which has experienced extensive oyster mortality starting in 1974; at the outfall from the Tenneco plant near Chestertown and in the Tenneco holding pond near the pond's outlet (latitude 39°15'00"N, longitude 76°02'30"W); at the outfall from the Chestertown sewage treatment plant at the edge of Morgan Creek (latitude 39°12'00"N, longitude 76°04'15"W); and at the Campbell factory near Chestertown (latitude 39°14'03"N, longitude 76"12'21"W). Three sampling excursions were taken in the period September 1977 through July 1979.

For comparison, some samples were taken in Baltimore Harbor (latitude 39°13'57"N, longitude 76°30'16"W), which was expected to be polluted with heavy metals, and other samples were taken near Tilghman Island (latitude 39°40'64"N, longitude 76°23'16"W), an area expected to be relatively free of pollution by heavy metals. Samples from the two Chester River sites, from Baltimore Harbor, and from the site near Tilghman Island were considered estuarine samples; samples from other sites were considered freshwater samples.

At each site, water temperature, pH, salinity, and dissolved oxygen were determined. Methods are given in Appendix C.

Water samples were taken with a Kemmerer bottle. Concentrated HCl (0.5 ml) was added to each 200-ml water sample to keep metals in solution. This brought the pH to 1.8 to 1.9. Prior to chemical analysis, the pH of each sample was adjusted to 2.0 with 1 N NaOH. Sediment samples were collected with a Van Veen dredge. A plastic corer was used to obtain material which had not touched the metal walls of the dredge. Samples were taken from the top centimeter of the core, and the remainder of the core was sliced into 1-cm bands which were frozen and will be maintained frozen for possible future use.

Samples from the sewage treatment plant, from the Campbell factory, and from the Tenneco plant were iced in the field and stored in ice until they were used in the laboratory. Samples taken on board ship (Buoy Rock, Spaniard Bar, Baltimore Harbor, Tilghman Island) were used for microbiological analysis within 15 min of sampling; the remainder of each sample was stored on ice until it was used in the laboratory.

Microbiological Samples

Water samples were used to prepare appropriate dilutions for plating. For sediment samples, 1.0 g (wet weight) was suspended in 9.0 ml of sterile estuarine salts; further dilutions were prepared from this suspension.

Total viable counts of aerobic, heterotrophic bacteria were made using the spread plating technique, plating on Nelson's medium (Nelson et al. 1973). Nelson's medium contains casamino acids; 5.0 g; yeast extract, 1.0 g; glucose, 2.0 g; agar, 15.0 g; and salt solution, 1 liter. For sediment samples from estuarine sites, the estuarine salts solution contained NaCl, 10.0 g; $MgCl_2 \cdot 6H_2O$, 2.3 g; KCl, 0.3 g; and distilled water, 1 liter. For samples from freshwater sites, the salts solution was used at one-tenth strength.

For viable counts of tin-resistant organisms, appropriate dilutions were spread on the surface of Nelson's medium prepared as above, supplemented with $SnCl_4$ to yield 75 ppm tin suspended in the medium. Extensive preliminary testing (Table 19) indicated that this concentration of tin was appropriate to select for tin-resistant organisms. Addition of $SnCl_4$ to the medium resulted in a fine precipitate of SnO_2 which was uniformly suspended in the medium by agitation. In one series of experiments the organisms resistant to organotin were estimated by plating on Nelson's medium containing 15 ppm tin as $(CH_3)_2SnCl_2$.

All platings were prepared in triplicate. Plates were incubated at 25 ± 2 C for 3 days prior to counting time.

Two systems were employed to determine if the microbial flora in samples could transform tin to volatile organotin compounds:

i) Bioflasks. 250-ml flasks containing 20 ml of Nelson's medium supplemented with 75 ppm tin as $SnCl_4$, were inoculated with 1.0 ml of sediment suspended in estuarine salts. Each flask contained a 10-ml beaker embedded in the agar. The beaker held 5.0 ml of a solution of 8 percent (wt/vol) citric acid in 10 percent HCl. The flask was sealed with a rubber stopper. After 14 days incubation at 27 ± 2 C, material in the beaker was examined for tin. Thus, if organisms growing on the medium produced volatile tin compounds, they would be detected in the acid solution. Sterile controls to check for nonbiological production

TABLE 19. DETERMINATION OF A CONCENTRATION OF TIN WHICH WOULD SELECT FOR TIN-RESISTANT MICROORGANISMS

Type of Sample	Tin added (ppmSn, as $SnCl_4$)	Viable count (mean ± standard error)*
Water	0	$8.7 \times 10^2 \pm 1.8 \times 10^2$ a
	50	$5.4 \times 10^2 \pm 6.8 \times 10^1$ b
Sediment	0	$8.1 \times 10^5 \pm 2.6 \times 10^4$ a
	50	$9.4 \times 10^4 \pm 8.5 \times 10^3$ b
	100	$4.0 \times 10^4 \pm 3.9 \times 10^3$ c
	150	$2.6 \times 10^4 \pm 7.1 \times 10^3$ c
	200	$1.7 \times 10^4 \pm 3.4 \times 10^3$ c

* Means with the same superscript are not significantly different at the 5 percent level as determined by a one-way analysis of variance (ANOVA).

of volatile tin compounds were included in each experiment. Each experiment also included positive controls in which the medium contained 75 ppm tin as dimethyltin chloride. The use of a solid medium containing a suspension of tin renders this method qualitative, not quantitative.

ii) Hungate tubes. Each tube (Hungate 1969), containing 5.0 ml of liquid Nelson's medium (Nelson's medium minus agar), was inoculated with 1.0 ml of sediment suspended in estuarine salts. Sterile controls and positive controls were included in each experiment. Tubes were incubated for 16 days at 27 ± 2 C on a rotary shaker operating at 80 rpm; each tube was then sampled for the presence of organotin compounds.

For each water or sediment sample, one set of bioflasks and Hungate tubes was inoculated from a sample which had received 500 µg of sodium azide per ml. This additional set of controls was used as a demonstration that effects observed in cultures were due to biological activity.

Analyses of Tin

Inorganic tin--

For sediment samples, 1.0 g (wet weight) was transferred to an acid-cleaned, screw-capped tube. Then, 2.0 ml of a solution containing 50 percent (vol/vol) HCl and 50 percent (vol/vol) concentrated HNO_3 were added. The tube was shaken vigorously for 1 hr. It was then centrifuged and 1.0 ml of the supernatant fluid was removed for analysis. The method of additions (Perkin-Elmer 1977) was used to minimize matrix interference.

Water samples (100 ml, not filtered) were adjusted to pH 2. A 15.0-ml quantity was then extracted with 10 ml of 15 percent (wt/vol) ammonium pyrrolidine dithiocarbamic acid (APDC) in distilled water. The APDC solution was prepared by dissolving APDC in distilled deionized water which had been adjusted to pH 7 with NaOH. The APDC solution was then extracted three times with CCl_4 to remove impurities. The water sample, containing APDC, was stirred for 10 min. It was then extracted with a minimum volume of methyl isobutyl ketone (MIBK). The mixture was agitated for 1 min and then allowed to separate for 10 min. The MIBK phase was analyzed for tin.

Organic tin--

The organic acid solution from bioflasks was analyzed directly for tin content.

Medium from Hungate tubes was extracted in such a way that inorganic tin compounds remained behind while organotin compounds were extracted. Extensive preliminary experiments showed that this extraction was effective. Medium was centrifuged at 3000 x g for 10 min to remove cells and sediment. The supernatant medium was then extracted with 2.0 ml of dichloromethane: chloroform (9:1, vol/vol). The extraction was conducted over a period of 1 hr, with periodic agitation. The mixture was then centrifuged at 1,000 x g for 10 min to sediment remaining inorganic tin precipitate. The lower, organic phase was removed and evaporated by dryness under a stream of nitrogen. The residue was dissolved in MIBK and analyzed for tin. Evaporation of the organic phase under nitrogen may have removed some organotin material, since organotins are volatile. Thus, results from Hungate tubes are qualitative, not quantitative.

Atomic absorption spectrophotometry--

Tin was analyzed with the aid of an HC-2200 graphite furnace with ramp accessory on a Perkin-Elmer model 503 AA unit using D_2 background correction (to minimize interference from "smoke" and nonatomic absorption) and an Sn electrodeless discharge lamp (EDL). Argon was the sheath gas.

Mineral acid solutions from sediment samples and organic acid solutions from bioflasks were analyzed with the EDL set at 286.3 nm. Samples were dried at 105 C for 40 sec (10 sec ramp), charred at 800 C for 40 sec (10 sec ramp), followed by atomization using maximum power at 2700 C for 8 sec with a 3-sec stop of gas.

MIBK from water samples and from biotubes was analyzed with the EDL set at 224.5 nm. Samples were dried at 110C for 40 sec

(10 sec ramp), charred at 650 C for 40 sec (no ramp), followed by atomization at 2500 C for 8 sec with a 3-sec stop of gas.

RESULTS AND DISCUSSION

Partially Developed Methods

One of the objectives of this work was to apply quantitative methods developed for other metals and organometallic compounds to tin and organotin compounds. The approach was to separate organotin compounds from one another via gas liquid chromatography (GLC) and to allow the effluent from the GLC to pass through a heated transfer tube to an atomic absorption spectrophotometer (AA) which would detect tin. Thus, those compounds which contained tin would be detected by the AA. The method is in use in several laboratories for other metals (Brinckman et al. 1976, Parris et al. 1977, Trachman et al. 1977) and contact was maintained with workers at the National Bureau of Standards throughout the project. Due to time pressures, further development of the method was stopped before it was ready for use on this project. Success was achieved in separating mono-, di-, tri-, and tetra-methyltin via GLC, although the system is not yet sensitive enough for direct application to environmental samples. The AA unit is close to being used as a detector for organotin compounds eluted from the GLC. Not the least of the difficulties was an 11-month delay in receiving the heated transfer line from the sole manufacturer. Work on this aspect of the project is continuing and support of this contract will be acknowledged in all publications.

Physical and Chemical Data on Samples

Physical and chemical data for samples taken on cruises and excursions in April 1978 and July 1979 are summarized in Tables 20 and 21. Temperatures and salinities were as expected for these sites in spring and summer seasons, respectively. pH values, which were taken only for the summer 1979 samples, were significantly higher at the freshwater sites than at the estuarine sites; they were highest at the two Tenneco sites. Values for dissolved oxygen were higher in April 1978 than in July 1979, as expected for spring and summer seasons, respectively. It is noteworthy that in summer 1979, dissolved oxygen was dangerously low near the bottom at the Buoy Rock site, which did not suffer extensive oyster mortality; while dissolved oxygen was only slightly higher at Spaniard Bar. Both oyster bars gave lower summer readings for dissolved oxygen than were found in Baltimore Harbor. A very high reading was obtained at the Tenneco pond in July 1979. A comparison of physiochemical data for the healthy bar, Buoy Rock, and the data for Spaniard Bar, show only dangerously low dissolved oxygen near the bottom as a potentially lethal condition. None of the physiochemical data from the freshwater sites gives a direct clue to the extensive kill of

TABLE 20. PHYSICAL DATA FOR APRIL 1978 CRUISE/EXCURSION

Station/Site	Depth* (meters)	Temperature (° C)	Salinity (°/oo)	Dissolved Oxygen (parts per thousand)
Buoy Rock	1	10.8	4.4	10.6
	6	11.7	5.2	10.7
	12	7.7	8.1	10.2
Spaniard Bar	1	12.3	4.6	10.3
	5	12.0	5.5	10.3
	10	12.1	5.4	10.2
Tenneco - effluent	-	20	3	9.1
Tenneco - pond	0.2	17	2	10.1
Campbell plant	0.2	18	1	7.0
Chestertown sewage treatment plant	0.2	19	1	9.4

TABLE 21. PHYSICAL DATA FOR JULY 1979 CRUISE/EXCURSION

Station/Site	Depth* (meters)	Temperature (° C)	pH	Salinity (°/oo)	Dissolved Oxygen (parts per thousand)
Buoy Rock	1	22.6		6.5	6.2
	6	22.5		6.6	6.0
	12	22.0	6.6	6.8	4.5
Spaniard Bar	1	24.0		5.6	7.4
	5	23.9		5.6	7.4
	10	23.9	6.1	5.6	7.3
Baltimore Harbor	1	23.2		3.5	7.9
	2.5	22.8		3.5	8.0
	5	22.7	5.2	3.8	7.6
Tilghman Island	1	22.5		8.7	9.4
	5	22.6		8.8	9.6
	10	22.6	5.5	9.1	9.3
Tenneco - effluent	-	30.0	9.6	2.0	8.4
Tenneco - pond	0.2	24.9	8.4	2.0	>20
Campbell plant	0.2	21.0	7.2	2.0	9.5
Chestertown sewage treatment plant	0.2	24.0	7.6	2.0	9.4

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* The deepest reading at each point was taken at approximately 1 m from the bottom.

benthic fauna in the Chester River. It is interesting that dissolved oxygen values for Baltimore Harbor are lower than for the site at Tilghman Island and only slightly higher than the values at Spaniard Bar.

Data for Microbial Populations

Data for enumeration of bacteria are shown in Tables 22 and 23. Counts in sediment are higher than counts in water, as expected. In general, estuarine sites showed higher bacterial counts in summer than in spring; this is expected as a function of temperature and of increased production of organic materials in the estuary in the summer.

TABLE 22. VIABLE COUNTS OF BACTERIA FROM APRIL 1978 CRUISE

Station/Site	Type of Sample	Total Viable Sample	Resistant to Inorganic-Sn	% Resistant
Buoy Rock	Water	1.2×10^{2}	1.6×10^{2}	133
	Sediment	2.0×10^{5}	1.3×10^{4}	6
Spaniard Bar	Water	$<1.2\times10^{2}$	$<1.2\times10^{2}$	-*
	Sediment	2.4×10^{5}	1.2×10^{5}	50
Tenneco - effluent	Water	2.8×10^{4}	2.0×10^{4}	10
Tenneco - pond	Water	1.1×10^{4}	2.2×10^{3}	20
	Sediment	2.4×10^{6}	3.7×10^{5}	16
Campbell plant	Water	6.4×10^{3}	3.2×10^{3}	50
	Sediment	4.1×10^{7}	2.1×10^{7}	51
Chestertown sewage treatment plant	Water	1.4×10^{2}	$<1.2\times10^{2}$	-*
	Sediment	5.8×10^{5}	3.2×10^{5}	55

* Data not accurate enough to yield a useful percentage.

TABLE 23. VIABLE COUNTS OF BACTERIA FROM JULY 1979 CRUISE

Station/Site	Type of Sample	Total Viable Count	Number Resistant to		% Resistant to	
			Inorganic-Sn	Organic-Sn	Inorganic-Sn	Organic-Sn
Buoy Rock	Water	5.0×10^1	$<1.5 \times 10^1$	0		0
	Sediment	2.3×10^6	3.4×10^5	1.6×10^4	15	1
Spaniard Bar	Water	7.3×10^2	$<1.5 \times 10^1$	0		0
	Sediment	7.8×10^5	2.8×10^5	1.4×10^4	36	2
Baltimore Harbor	Water	3.3×10^4	9.5×10^3	5.8×10^2	29	17
	Sediment	6.0×10^5	2.3×10^5	1.3×10^4	40	2
Tilghman Island	Water	3.1×10^3	1.5×10^1	0	<0.5	0
	Sediment	3.2×10^5	6.0×10^4	0	19	0
Tenneco - effluent	Water	1.2×10^5	4.0×10^4	1.1×10^4	33	9
Tenneco - pond	Water	1.5×10^4	7.2×10^3	6.6×10^3	50	4
	Sediment	9.2×10^5	1.1×10^5	9.5×10^4	12	10
Campbell plant	Water	1.3×10^2	$<1.5 \times 10^1$	0	<8	0
	Sediment	8.1×10^6	9.3×10^5	5.2×10^5	12	6
Chestertown sewage treatmemt plant	Sediment	5.6×10^6	3.1×10^6	5.5×10^5	55	10

In most cases, a significant fraction of the microbial population was resistant to inorganic tin and is, therefore, potentially capable of metabolizing tin to more toxic compounds. A much smaller fraction of the population was resistant to the organotin compound, dimethyltin chloride, attesting to the antibacterial properties of organic tin compounds. The data are consistent with the hypothesis that aquatic microorganisms can protect themselves against toxic tin compounds by transforming tin to organotins which, although toxic in themselves, are volatile and leave the immediate vicinity of the cell which formed them.

Sediment from Spaniard Bar did not contain higher numbers of tin-resistant organisms than sediments from Buoy Rock, although a higher percentage of the population was resistant to tin at Spaniard Bar than at Buoy Rock. Sediment samples taken at the Chestertown sewage treatment plant contained a high percentage of organisms resistant to inorganic tin. There is little indication of a higher level of tin-resistant microflora at Spaniard Bar than at Buoy Rock. The data available do not suggest that the freshwater sites contain higher numbers of tin-resistant organisms than the estuarine sites.

Data for production of volatile tin compounds are summarized in Table 24. The method used was effective in detecting organic tin compounds produced in bioflasks, as indicated by values observed from sterile medium which contained dimethyltin chloride. Variation among replicates indicates that the method is qualitative. Flasks which received inoculum containing the metabolic poison sodium azide yielded no volatile tin, indicating that volatile tin detected was the result of biological activity. Results from bioflasks and Hungate tubes demonstrate that each site contains microorganisms capable of converting inorganic tin to volatile organotin(s). The species of organotin produced were not identified.

Tin in Water and Sediments

The lower limit of sensitivity for tin analysis was 2 ppb. A recovery value of 77.8 percent was obtained when sediment from Tilghman Island was spiked with $SnCl_4$. When salt water or Nelson's liquid medium was spiked with $SnCl_4$, recoveries of 94-96 percent were obtained.

Sediment samples contained more tin than water samples (Table 25), as expected. Sediment from Baltimore Harbor, known as a polluted site, contained over 200 ppm tin (>0.02 percent on a wet weight basis). In contrast, sediments from the Tilghman Island site contained less that 1 ppm (<0.0001 percent on a wet weight basis). All sediments associated with the Chester River, including sediments from the three freshwater sites, yielded more tin than sediments from the Tilghman Island site (Table 25).

TABLE 24. PRODUCTION OF VOLATILE TIN IN MEDIA INOCULATED WITH SEDIMENT

Source of inoculum	Result from		
	Bioflask		Hungate tube
	Replicate No.	µg Sn in organic acid solution*	
None, sterile control with medium containing dimethyltin chloride	1	148	++++†
	2	45	
Buoy Rock	1	21	+
	2	0	
Spaniard Bar	1	262	++
	2	57	
Baltimore Harbor	1	344	+
	2	145	
Tilghman Island	1	658	+
	2	0	
Tenneco pond	1	361	++
	2	314	
Campbell plant	1	218	+
	2	144	
Chestertown sewage treatment plant	1	176	++
	2	201	

* Corrected for values obtained from sterile flasks containing inorganic tin.

† The notation used represents peak height from the atomic absorption unit: -, 0.0; +, 0.1 to 0.2; ++, 0.3 to 0.6; +++, 0.6 to 1.0; ++++, 1.0 and above.

TABLE 25. TIN IN WATER AND SEDIMENT SAMPLES

Station/Site	Type of Sample	µg/ml or µg/g for samples taken in April 1978 Mean	April 1978 Standard error	July 1979 Mean	July 1979 Standard error
Buoy Rock	Water	<0.002	-	<0.002	-
	Sediment	1.574	0.180	7.882†	0.088
Spaniard Bar	Water	0.240	0.007	<0.002	-
	Sediment	9.686	0.420	3.061‡	0.187
Baltimore Harbor	Water			<0.002	-
	Sediment			239.633*	14.073
Tilghman Island	Water			<0.002	-
	Sediment			0.861§	0.052
Tenneco - effluent	Water	0.034	0.002	0.052	0.002
Tenneco - pond	Water	0.103	0.005	0.023	0.001
	Sediment	0.043	0.081	3.523‡	0.046
Campbell plant	Water	0.032	0.001	<0.002	-
	Sediment	1.583	0.131	3.876‡	0.028
Chestertown sewage treatment plant	Water	0.002	-	0.152	0.028
	Sediment	2.857	0.060	5.178†,‡	0.028

*, †, ‡, §. Sediment samples values with identical superscripts were not significantly different at the 5 percent confidence level.

Smith and Burton (1972) reported values of <0.05 to 16 ppm in various marine sediments. Surface sediments from Narragansett Bay contain 20 ppm tin (Hodge, Seidel, and Goldberg 1979). Furr et al. (1976) reported values as high as 492 ppm tin in sewage sludge. Thus, values obtained for sediments in the present study are in the same range as reported by other workers. Sediments in the Chester River, in the Tenneco holding plant, near the Campbell plant, and in the Chestertown sewage treatment plant all contain significant quantities of tin (Table 25). Based on the two samples taken, Spaniard Bar, which suffered an oysterkill, did not yield significantly more tin than Buoy Rock, which did not suffer such a kill (Table 25). The null hypothesis that mean tin concentration (Spaniard Point) did not differ from the mean tin concentration (Buoy Rock) was supported. Thus, it is not possible to attribute the oysterkill at Spaniard Bar solely to pollution by tin, although interaction of tin with other factors cannot be excluded. It is also possible that different tin species are present at the two sites, e.g. inorganic tin vs. one or more organotin species.

Smith and Burton (1972) reported inorganic tin concentrations of 0.02 to 0.04 μg/kg (0.002 to 0.04 parts per trillion) in estuarine and continental shelf waters. Hodge et al. (1979) reported 2 to 38 ng inorganic tin/liter (0.002 to 0.038 μg/kg, 0.002 to 0.038 parts per trillion) in water from San Francisco Bay and San Diego Bay, and 84 to 490 μg/liter (0.084 to 0.490 μg/kg, 0.084 to 0.490 parts per trillion) in water from Lake Michigan. In Lake Michigan water, the concentrations of organic tin compounds were two to eight times greater than the concentrations of inorganic tin. The methods used in these two studies were several orders of magnitude more sensitive than the method employed in the present work. Some of the values obtained for water samples in the present work (Table 25) are significantly higher than values reported by Smith and Burton (1972) and by Hodge et al. (1979). Variations from one sample time to another (e.g., values for water from Spaniard Bar and water from the Tenneco pond) can be expected due to variations in input and in flow rate, which can also account for the low value in the water sample from Baltimore Harbor. Our data suggest that water in the Chester River and water entering the Chester River from the Tenneco plant, from the Campbell plant, and from the Chestertown sewage treatment plant sometimes contains significant quantities of tin. The chemical species of tin were not identified in the present study.

The data obtained in the present study yielded reasonable recovery from spiked samples. The method of additions was used successfully to alleviate matrix interference. Thus, the values obtained for tin are regarded with confidence, even though they are high.

Statistical Analyses

Data for bacterial enumerations, and data for tin levels were analyzed using a one-way analysis of variance (ANOVA). Sample means were compared using the studentized (SNK) multiple range test.

Correlation coefficiencts (r) were analyzed for significance of association between tin concentration and counts of tin-resistant microorganisms; and for significance of association between tin concentration and percent of the microbial population resistant to tin. All associations were tested at the 5 percent level of significance. No significant positive or negative correlations were noted. Thus, the data available do not suggest that tin (organic and/or inorganic) selects for a population of tin-resistant microorganisms.

General Discussion

The data indicate that surface sediments from oyster bars in the Chester River contain significantly more tin than is contained in sediments from a site near Tilghman Island, but they contain significantly less tin than sediment from Baltimore Harbor. The three freshwater sites examined--the Tenneco plant, the Campbell factory and the Chestertown sewage treatment plant--all contribute tin to the Chester River ecosystem at an undetermined rate. There may be other contributors. It is clear that microorganisms capable of converting inorganic tin to more toxic organotin compounds are ubiquitous in the Chesapeake ecosystem (Table 24).

Zuckerman et al. (1978) reviewed the known toxic effects of organotin compounds on a variety of living organisms. Relatively little is known of effects on estuarine organisms, but the following toxic levels have been reported for organisms relevant to the present study: guppies, less than 1 ppm of bis(tributyltin)oxide and 0.1 ppm triphenltin hydroxide; molluscs, 1.0 ppm of several trialkyltin and 0.05 - 0.10 bis(tributyltin)oxide; algae, barnacles, shrimp and tubeworms, 0.1 to 1.0 ppm of tributyl and triphenyltin compounds. Very little is known of the levels of organotin compounds in estuarine systems. But sediments in the Chester River ecosystem contain between 3 and 8 ppm of tin (Table 25). Data from the present study do not indicate the chemical speciation of the tin compound(s) detected. Although the chemical species of tin compound(s) was not establised, if even one-third of the tin is present as organotin compounds, the estuarine biota could be at risk, particularly if stress from toxic tin(s) is coupled with other stresses such as low dissolved oxygen.

Comparisons of tin in surface sediments from Spaniard Bar with data from Buoy Rock and with data from other marine sediments (Smith and Burton 1972, Hodge et al. 1979) indicate clearly that tin is not the sole source of the oysterkill at Spaniard Bar. However, if oysters and other benthic organisms were stressed by other factors, e.g. low dissolved oxygen, tin could contribute additional stress leading to death.

Little is known about bioaccumulation of tin by benthic invertebrates. Until recently, analytical methods were not sensitive enough to permit one to deal with tin in the same quantitative manner as with other inorganic pollutants such as copper, cadmium, mercury, and zinc. These include aspects of levels of tin and organotins in the environment, accumulation of tin compounds in the food chain, chemical and biological formation and transformation of inorganic tins and organotins, transport of tin compounds in soils and in aquatic ecosystems, and toxicity of tin compounds to the biota. The appropriate questions should be asked regarding tin. Moreover, it is possible that the gut flora of oysters and other invertebrates can convert inorganic tin to more toxic organotins; these questions should be addressed.

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APPENDIX A

CHESTER RIVER OYSTER MORTALITY

A review of recent Fisheries Administration catch records and oyster bar surveys of the upper Chester River indicates a significant decline in the populations of both oysters and associated organisms.

A major oyster mortality occurred during the spring and summer of 1973 in Langford Creek and the Chester River above Oldfield oyster bar. The 1974 fall survey showed the mortality was continuing as Oldfield had died-off and some mortality had occurred on Piney Point bar. No live oysters were found above Hells Delight bar during the 1975 oyster survey. These mortalities caused oyster landings from the area above Piney Point bar to decline from 50,000 bushels in 1972-1973 to 650 bushels for the 1975-1976 season.

The following is the text of a 8 June 1978 letter sent to us by George E. Krantz of the Horn Point Environmental Laboratories:

"Please excuse the long delay in sending you some data on the Chester River mortality. Unfortunately my files were loaned to other investigators who removed many of the original documents that I described to you during our phone conversation last month. I think I have found copies of most of the information but I was unable to find a complete briefing document for you. Perhaps some of the data, especially from the Dept. of Natural Resources will be helpful in your study. At a later date we may be able to more thoroughly discuss the observations that may have existed in this historical phenomenon."

Data on pages 103 thru 110 were compiled by Roy Scott, DNR, from field data sheets reflecting Fall oyster bar survey results. Time of survey of specific bars varied from October thru March of a given year.

1970. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*
Ferry	216/75	90/8	2	4
Ferry	174/65	236/30	2	8
Side Shoal	42/20	762/80	2	2
Buoy Rock	198/70	74/15	28	2
Buoy Rock	188/60	43/15	4	0
Buoy Rock	110/55	40/20	0	6
Blunt	50/9	0	2	58
Blunt	180/65	244/30	0	0
Blunt	266/90	12/4	4	0
Hail Point	114/50	208/30	6	2
Hail Point	170/70	26/5	0	0
Hail Point	182/85	12/4	0	2
Hail Point	98/60	12/2	2	2
Carpenters Island	124/40	530/55	4	4
Carpenters Island	236/75	28/3	4	4
Durdin	98/30	406/45	4	10
Durdin	124/40	152/50	8	10
Durdin	36/40	22/1	2	2
Horserace	182/95	6	2	0
Horserace	110/45	414/40	4	0
Piney Point	202/70	38/10	0	4
Bay Bush Point	160/70	56/10	6	12
Hells Delight	158/60	4	0	0
Hells Delight	282/90	4	0	4

(continued)

1970. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*
Bluff Point	212/80	28/2	0	6
Middlegrounds	230/85	16/2	0	10
Oldfield	4	686/85	0	16
Oldfield	216/75	16/3	0	8
Oldfield	156/50	22	6	0
Willow Bottom	116/55	10/2	0	10
Hudson	186/50	30/6	4	8
Hudson	174/30	494/60	0	8
Boathouse	78/25	234/35	0	6
Drum Point	55/35	20/3	0	0
Davis Creek	142/60	30/3	0	4
Ebb Point	100/45	306/38	4	2
Ebb Point	132/40	117/12	2	2
Spaniard Point	148/30	730/60	0	4
Spaniard Point	4	908/100	0	0
Cliff	72/30	400/60	0	6
Emory Hollow	20/15	2	0	2
Mummys Cove	116/65	4	2	4
Shippen Creek	132/70	0	0	4
Shippen Creek	170/50	18/3	0	16

* Number/Percentage of 1 oyster bushel.

1973. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*	Associated Organisms Present †
Buoy Rock	152/70	80/10	0	0	0
Hail Point	162/90	22/4	2	2	X
North of Hail Pt.	108/50	36/7	0	4	X
Carpenters Island	114/50	16/2	0	4	X
Durdin	188/65	80/7	0	8	X
Horserace	118/50	58/10	0	8	0
Piney Point	54/30	0	0	12/6	X
Bay Bush Point	112/55	8/<1	0	18/8	X
Bluff Point	54/40	2	0	20/25	X
Oldfield	12/8	2/<1	0	52/40	X
Oldfield	104/70	2	0	10/4	X
Oldfield	128/60	12/<1	0	14/6	X
Oldfield	58/40	8/<1	0	20/23	X
Oldfield	58/40	38/10	0	46/12	0
Oldfield	16/5	4	0	60/40	X
Willow Bottom	0	0	0	122/85	X
Nichols	0	0	0	126/75	X
Holton Point	0	0	0	82/55	X
Ebb Point	2	0	0	64/45	X
Spaniard Point	0	2	0	142/45	X
Spaniard Point	0	0	0	154/80	X
Cliff	0	2	0	130/50	X
Cliff	0	0	0	112/45	X

(continued)

1973. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*	Associated Organisms Present †
Sheep	0	0	0	26/20	X
Mummy Cove	0	0	0	50/25	X
Shippen Creek	0	0	0	96/25	X

* Number/Percentage of 1 oyster bushel.

† O = no organisms detected; X = organisms present.

1974. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*	Associated Organisms Present †
Buoy Rock	184/80	102/16	0	2	0
Blunt	134/55	4/1	0	12/3	X
Hail Point	90/40	6	0	20/6	X
Carpenters Island	80/30	6/1	0	14/2	X
Durdin	108/53	12/1	0	26	X
Horserace	72/40	4/<1	0	10/10	X
Piney Pt. (Lower)	90/40	38/8	0	22/5	X
Piney Pt. (Upper)	84/30	10/1	0	46/15	X
Bay Bush Point	56/20	72/15	0	22/5	X
Hells Delight	10/5	0	0	16/10	X
Bluff Point	54/40	2	0	20/25	X
Oldfield	0	0	0	68/25	X
Willow Bottom	0	0	0	74/25	X
Nichols	0	0	0	30/20	X
Sand Thistle	0	0	0	60/15	X
Boat House	0	0	0	88/60	X
Drum Point	0	0	0	28/12	X
Holton Point	0	0	0	82/55	X
Ebb Point	0	0	0	68/25	X
Spaniard Point	0	0	0	132/50	X
Cliff	0	0	0	60/25	X
Emory Hollow	0	0	0	0	X

(continued)

1974. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*	Associated Organisms Present †
Emory Hollow	0	0	0	0	X
Mummys Cove	2	0	0	30	0
Mummys Cove	0	0	0	42/25	X
Shippen Creek	2	0	0	22/10	X
Shippen Creek	0	0	0	56/40	X

* Number/Percentage of one oyster bushel.

† 0 = no organisms detected; X = organisms present.

1975. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*	Associated Organisms Present +
Strong Bay	162/60	4/<1	0	8/4	X
Ferry	170/80	6/<1	0	16/8	X
Ferry	84/15	0	0	20/6	X
Ferry	184/90	6	0	10/6	X
Side Shoal	196/90	12/4	0	8/4	0
Buoy Rock	150/65	70/12	0	2/<1	X
Blunt	204/85	0	0	16/10	X
Blunt	128/50	4/1	0	20/12	X
Blunt	108/55	4/<1	0	12/6	X
Hail Point	188/80	14/2	0	8/4	X
North of Hail Pt.	148/45	16/1	0	10/8	X
Carpenters Island	122/45	20/5	0	30/12	X
Carpenters Island	160/50	22/4	0	10	X
Carpenters Island	118/75	4/1	0	4/2	X
Carpenters Island	140/45	54/11	0	14/12	X
Carpenters Island	238/50	21/6	0	0	X
Horserace	138/60	24/3	0	6/3	X
Horserace	138/50	60/12	0	19	X
Piney Point	70/25	102/18	0	8	X
Piney Point	118/50	54/10	0	30/25	X
Bay Bush Point	8	52	0	14	0
Oldfield	0	0	0	5	0
Oldfield	0	0	0	15	X

(continued)

1975. Continuation of ltr. from G. E. Krantz of June 8, 1978.

Bar	Markets*	Smalls*	Spat*	Boxes*	Associated Organisms Present †
Oldfield	0	0	0	15	X
Willow Bottom	0	0	0	3	X
Nichols	0	0	0	10	X
Drum Point	0	0	0	5	0
Holton Point	0	0	0	48/20	X
Spaniard Point	0	0	0	94/22	X
Cliff	0	0	0	18/6	0

* Number/Percentage of 1 oyster bushel.

† 0 = indicates no organisms detected; X = organisms present.

APPENDIX B

ACCOUNT OF INTERLABORATORY TESTS ON SPLIT SAMPLES

This is a chronological account of interactions with Environmental Monitoring and Support Laboratory (EMSL), Office of Research and Development, U.S. Environmental Protection Agency, Cincinnati, Ohio. Contact: William L. Budde.

October 1978

Two dried sediment samples were each split and respective subsamples were sent to EMSL in Cincinnati. They were labeled: (1) Chester River, mouth, top sediment; (2) Tenneco Pond sediment (>100 ppm dioctyl phthalate). The EMSL facility was selected due to its fine reputation in the scientific community.

January 1979

EMSL advised this laboratory of their results on the two samples: (1) 61 ppm of DEHP in the Chester River, mouth, and (2) 1700 ppm DEHP in the Tenneco Pond. No error values nor blank values were given. In a telephone conversation prior to this report, EMSL communicated a preliminary verbal report indicating 6 ppm of DEHP in the Chester River, mouth sediment. (After discovering a computational error, the corrected value was described as 58 ppm.) The value this laboratory had determined--prior to receiving the EMSL report--was 0.6 ppm DEHP in Chester River, mouth sediment and 1200 ± 100 ppm DEHP in Tenneco Pond sediment.

Due to the large discrepancy (2 orders of magnitude) this laboratory undertook an extensive battery of experiments to explore previously undetected systematic errors, and to find a possible reason for its low value for DEHP in Chester River, mouth sediment. After 2 months of experimentation, we continued to obtain values less than 1 ppm. In fact, improvement of our methodology produced a value lower than determined previously 0.1 ppm.

April 1979

Agreement for retrial was secured and a second set of split samples was sent to EMSL Cincinnati. This time three samples were sent: (1) Dry Chester River mouth sediment, (2) Wet Chester River mouth sediment, which EPA dried themselves, and (3) "organic free" clay which we had carefully spiked with DEHP.

June 1979

EMSL returned the second set of results and reported a drastically lowered result for Chester River, mouth.

	EPA-Cincinnati	Univ. Maryland
(1) Chester River, mouth dried by U. of Maryland	0.3 mg/kg DEHP	0.097±0.03 ppm, DEHP
(2) Chester River, mouth dried by EPA	0.4 mg/kg DEHP	
(3) Attapulgite, spiked	121 mg/kg DEHP	200±10 ppm, DEHP

In a telephone conversation prior to this report, EMSL commented that during the time of the first exchange, EPA Cincinnati had been experiencing some contamination problems with their homogenizer used in sediment extraction. This is a probable explanation for the excessively high values found by EMSL in the original exchange. In the report of June 1979, signed by William Budde, it was mentioned that EMSL procedures were the same as before, except that they had used a new Tissumizer (R) by Tekmar.

While there is still some difference between the values determined by each laboratory in this study, we believe they are within acceptable limits especially considering the history of trace organic interlaboratory comparisons (Hilpert et al., 1978, Hertz et al., 1979).

Hertz, H. S., L. R. Hilpert, W. E. May, S. A. Wise, J. M Brown, S. N. Chesler, and F. R. Guenther. Special Technical Publication 686, American Society for Testing and Materials, 1979.

Hilpert, L. R., W. E. May, S. A. Wise, S. N. Chesler, and H. S. Hertz. Interlaboratory comparison of determinations of trace level petroleum hydrocarbons in marine sediment. Anal. Chem. 50:458-463, 1978.

APPENDIX C

METHODS FOR WATER QUALITY MEASUREMENTS IN TIN STUDY

Water temperature was measured with the temperature probe on the YSI oxygen meter used to determine dissolved oxygen. Measurement of pH was with a Coleman meter, and salinity was measured using a Wheatstone conductivity bridge.